S100P Is Selectively Upregulated In Tumor Cell Lines Challenged With DNA Cross-Linking Agents

By

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DEDICATION

To my parents,

my sisters,

for their unconditional and continual love, support and encouragement.

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LIST OF ABBREVIATIONS

AML	acute myeloid leukemia
BM	bone marrow
Ct	threshold cycle
D	day
DEE	2-Diethylaminoethyl chloride
DME	2-Dimethylaminoethyl chloride
FA	Fanconi anemia
g	gram
GST	glutathiones transferase
GSTA2	Glutathione S-transferase A2
HN2	nitrogen mustard
HPV	human papiloma virus
Hr	hour
ICC	immunocytochemistry
IHC	immunohistochemistry
IC50	inhibition of 50% cell growth
ICL	interstrand cross-links
MDR-AML	MDS related AML
MDS	myelodysplastic syndromes
MiCK	microculture kinetic assay
Min	minute
ml	milliliter
mM	millimolar
MMC	mitomycin C

NLS	nuclear localization signal
PBMCS	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PHA	phythamagglutinin
Real time PCR	real time polymerase chain reaction
RT	reverse transcription
S100P	S100 calcium-binding protein P
SCT	stem cell transplantation
Sec	seconds
SiRNA	small interfering RNA
SSC	standard saline citrate
TDN-AML	true de novo AML
μM	micromolar

I.

INTRODUCTION

MDS and MDR-AML

Myelodysplastic syndrome (MDS), also known as preleukemia, refers to a group of clonal disorders of unknown cause in which the bone marrow functions abnormally and fails to produce mature blood cells in adequate numbers. MDS affects the production of red blood cells, platelets, and neutrophils (Bennett, Catovsky et al. 1982; Bernell, Jacobsson et al. 1996). MDS progresses to acute myeloid leukemia (AML) in about 30 percent of patients and is directly fatal in most of the rest. Although MDS is most common in adults over the age of 50 (median age estimated at 65-70 years old), it also occurs in children and younger adults. There are four main types of MDS: refractory anemia, refractory anemia with ring sideroblasts, refractory cytopenia with multilineage dysplasia., and refractory anemia with excess blasts (Nosslinger, Reisner et al. 2001). Distinction between the subtypes of MDS is largely based on the proportion of blasts in the peripheral blood (PB) and bone marrow (BM), the degree of dysplasia in hematopoietic precursors, and the presence of clonal cytogenetic abnormalities. The BM blast count has a major influence on prognosis in MDS (Greenberg, Cox et al. 1997). There are no highly specific and sensitive diagnostic markers available for MDS. Allogeneic bone marrow transplantation (transfusion using marrow from a compatible donor) is currently the most effective form of treatment for most types of MDS. However, most patients have high risk for this procedure due to their advanced age; and even with this procedure, up to 30-40% of patients relapse (Bennett, Catovsky et al. 1982).

Approximately 50% of AML cases follow or are biologically related to MDS. The pathogenesis of MDS and subsequent MDS related AML (MDR-AML) is unknown, and it is not possible to consistently separate these AML cases from the remainder of cases, true de novo AML (TDN-AML). Accurate distinction between these disease sets would likely improve patient outcomes, since treatment differs substantially for MDR- versus TDN-AML (Head 1996). The cytogenetic differences and differing response to therapy of these two groups of AML appear to reflect the underlying biologic differences of the two diseases. TDN-AML is a chemotherapy-sensitive disease characterized by specific recurring translocations, whereas MDS and MDR-AML are characterized by numerous chromosomal abnormalities and are typically resistant to chemotherapy or other current treatment except for stem cell transplantation (SCT). Because of its age distribution (occurring in elderly people), most patients are ineligible for SCT (Head 2002).

The pathogenesis of MDS and MDR-AML is unknown, but some data suggest a relationship to interstrand cross-link (ICL) DNA damage (Dronkert and Kanaar 2001). One example is seen in therapy-related MDR-AML. Some cancer patients develop MDR-AML after they undergo chemotherapy treatment with DNA cross-linking agents (Anderson, Kopecky et al. 2002). Another example is found in MDS and MDR-AML that develop in patients with Fanconi Anemia, an inherited deficiency in repair of cross-link DNA damage. Thus, ICL DNA damage is implicated in the pathogenesis of at least some cases of MDS and MDR-AML (Auerbach 1992; Head 1996; Lensch, Tischkowitz et al. 2003). However, the repair system for correcting such damage is largely unknown (Islas, Vos et al. 1991; Vos and Wauthier 1991; Larminat, Zhen et al. 1993; Wiencke and Wiemels 1995; Lawley and Phillips 1996; Buchwald and Moustacchi 1998; Dronkert and Kanaar 2001).

Fanconi Anemia, DNA Cross-Linking Agents And MDS And MDR –AML

Fanconi anemia (FA) is an autosomal recessive disease, and affected patients present with a diverse assortment of congenital malformations, the most common being limb malformations and abnormal skin pigmentation (Auerbach 1992; Buchwald and Moustacchi 1998; Clarke, Marsh et al. 1998). Progressive bone marrow (BM) failure and its complications are the major causes of death of FA, and bone marrow transplant is currently the only curative treatment available. FA patients are also predisposed to developing cancer, particularly MDS and MDR-AML (Auerbach 1992; Buchwald and Moustacchi 1998).

Cells cultured from FA patients display an increased level of spontaneous chromosomal abnormalities when compared to normal cells, and this effect is amplified when the cells are exposed to DNA cross-linking agents such as mitomycin C (MMC). For this reason, FA has been classified as a chromosomal instability disorder. Cellular sensitivity to DNA cross-linking agents is currently used as a diagnostic test for FA (Pritsos and Sartorelli 1986; Tomasz 1995).

DNA Cross-Linking Agents

DNA cross-linking agents cause cross-links both inside each DNA strand (DNA intrastrand cross-links) and between two complementary strands (DNA interstrand cross-links). ICLs are among the most toxic of insults to DNA. A single ICL can kill repair-deficient bacteria and yeast, and about 40 ICLs can kill repair-deficient mammalian cells (Lawley and Phillips 1996). Moreover, ICLs can induce mutations and rearrangements of DNA by incorporation of DNA damage into the genome. Such mutation may result in some instances in acquisition of uncontrolled cell growth and tumor formation, as in MDS and MDR-AML. Many different agents are capable of inducing ICLs in the genome. A

well-known class of ICL-inducers is chemotherapeutic agents like MMC, cisplatin, nitrogen mustard, nitrosourea, and their derivatives.

Organisms have developed strategies to deal with DNA damage in order to survive. A number of specialized repair pathways have evolved, each of which processes specific kinds of DNA damage. An intriguing aspect of ICL repair is that several of these pathways apparently have to work together in order to remove or bypass an ICL (Larminat, Zhen et al. 1993; Chu 1994; Dronkert and Kanaar 2001). While the major ICL repair pathway in bacteria is well-characterized, both genetically and biochemically, ICL repair in eukaryotes is less well understood (Dronkert and Kanaar 2001).

ICL agents have different toxicities depending on a number of factors, including cellular uptake, metabolic activation and detoxification. Most ICL agents have to be transported into the cell and then into the nucleus. A number of ICL agents are metabolically converted in the cell into an agent with a much higher activity (Wiencke and Wiemels 1995). Metabolism also yields other damaging agents. Mitomycin C, for example, undergoes a cycle of oxidation and reduction, thereby activating the agent and generating reactive oxygen species that can cause additional damage to the cell (Pritsos and Sartorelli 1986; Tomasz 1995; Buchwald and Moustacchi 1998). Nitrogen mustard and cisplatin also give rise to reactive oxygen species (Clarke, Marsh et al. 1998). On the other hand, metabolism also inactivates ICL agents, often via the cytochrome P450 reductase system (Wiencke and Wiemels 1995). Furthermore, intracellular thiols, such as glutathione and metallothionein, can detoxify ICL agents (Chu 1994).

ICL agents form a number of adducts with DNA. These adducts are not always produced randomly throughout the genome, because chromatin structure can influence adduct formation (Vos and Wauthier 1991); (Islas, Vos et al. 1991; Larminat, Zhen et al.

1993). ICLs only represent a small fraction of the adducts formed by DNA cross-linking agents. Nevertheless, they are thought to be the main determinant of the toxicity of ICL agents, due to their inhibition of DNA strand separation and, therefore, of DNA replication, transcription, and segregation (Islas, Vos et al. 1991; Larminat, Zhen et al. 1993; Lawley and Phillips 1996).

To adequately respond to the ICLs, cells have to recognize them. They could recognize the ICL itself by its distortion of the DNA helix. However, the three-dimensional structures of ICLs are variable, which could influence the efficiency of ICL recognition and repair. Cells could also recognize the transcription or replication block induced by the ICLs. Any damage response will include cell cycle arrest and, if repair fails, cell death.

DNA Cross-Linking Agents and S100 calcium-binding protein P (S100P)

As mentioned earlier, ICL DNA damage caused by bifunctional alkylating agents is implicated in the pathogenesis of therapy-related MDS and MDR-AML. This is supported by the development of MDS and MDR-AML in Fanconi anemia patients and in patients who receive bifunctional cross-linking agent chemotherapy. Therefore, characterizing mammalian cells (specifically hematopoietic cells) responses to DNA cross-link damage may be relevant to elucidating the molecular pathogenesis of MDS and MDR-AML. To search for genes selectively involved in the cellular response to DNA cross-linking agents, HL-60 cells were treated with bifunctional cross-linking agents. Gene expression profiles were compared using cDNA microarray analysis. To eliminate the background noise caused by monofunctional adducts, cells were also treated with equitoxic doses of monofuctional alkylating agents, Dimethylaminoethyl chloride (DME) and 2-Diethylaminoethyl chloride (DEE) for comparison (Figure I-1). The comparison among non-treated HL-60 cells and

those treated with HN_2 or DME/DEE revealed that S100P mRNA was selectively upregulated in bifunctional alkylating agent treated HL-60 cells but not in non-treated control or cells treated with monofunctional agents.

S100P, a novel member of the S100 calcium binding protein family, present in human placenta, has 95 amino acid residues and shares about 50% sequence identity with the brain S100 proteins alpha and beta (Gribenko, Hopper et al. 2001; Sorci, Riuzzi et al. 2003). Unlike the rest of the family, most of which are located on chromosome 1, the human S100P gene is located on chromosome 4p16 (Donato 1991; Donato 1999; Donato 2001; Donato 2003). It has three exons, two of which encode calcium-binding domains, a common motif shared by the S100 calcium-binding protein family. Like other members of the S100 family, dimer formation appears to be crucial for function. S100P exists as a homodimer and has two functional EF hand/polypeptide chains. The S100P dimmer subunit contains four helices: helix 1 (residues 3–18), helix 2 (residues 30–40), helix 3 (residues 53–61), and helix 4 (residues 71–92). In S100P, the two calcium-binding loops cross in an antiparallel manner and cross-strand hydrogen bonds between 27–28 and 68–69, respectively. The hydrogen bonds between these two strands contribute to a tight interaction between the loops of the two EF-hands (Zhang, Wang et al. 2003). This small 95-amino acid protein binds divalent cations, including calcium and magnesium (Figure I-2). The binding has an effect on protein conformation, and in turn interaction with target proteins (Gribenko, Lopez et al. 1998).

The S100 class of EF-hand calcium-binding proteins is a multigene family expressed exclusively in vertebrates (Donato 2003). Members of this family can be upregulated during cell differentiation and malignancy (Schäfer & Heizmann, 1996; Grigorian et al., 1996), associated with chemotaxis (Newton & Hogg, 1998; Jinquan et al., 1996), and may have

functions in the cell cytoskeleton and motility (Donato, 1991). S100P was first cloned from placenta (Becker et al 1992), and the biological function and cellular localization of S100P are areas of active investigation. S100P upregulation has been observed in a wide range of cancers, suggesting a relationship to initiation and development of malignancies. S100P overexpression is associated with immortalization of human breast epithelial cells *in vitro* and early stages of breast cancer development *in vivo* (Guerreiro Da Silva, Hu et al. 2000; Mackay, Jones et al. 2003). The S100P protein and message are both regulated by androgens in prostate cancer cells, demonstrating different expression in androgen-dependent and androgen-independent prostate cancer cell lines (Averboukh, Liang et al. 1996). cDNA microarray analysis of the CWR22 xenograft model system found S100P to be upregulated in prostate cancer progression. This upregulation of RNA and protein was also seen in patient samples (Spyro Mousses 2002). S100P is transiently expressed during the early stages of differentiation in human esophageal epithelial cells and it is overexpressed in esophageal cancer (Sato and Hitomi 2002; Zhi, Zhang et al. 2003). In addition, S100P expression is upregulated in pancreatic cancer. Comparisons among pancreatic adenocarcinoma, pancreatic cancer cell lines, normal pancreas, and chronic pancreatitis reveal that S100P gene is selectively expressed in the neoplastic epithelium of pancreatic adenocarcinoma (Hasel, Rau et al. 2001; Logsdon, Simeone et al. 2003). In addition, the E7 oncoprotein of Human papillomavirus type 16 (HPV16), upregulates S100P. HPV16 is a dominant risk factor for the development of cervical cancer (Hellung Schonning, Bevort et al. 2000). Transgenically overexpressed exogenous S100P in NIH3T3 cells is secreted and can stimulate proliferation of NIH 3T3 cells (Arumugam, Simeone et al. 2003). Although upregulation of S100P has been observed in many tumors, at this time the contribution of S100P to human tumor development and progression is unknown.

Overview of Experimental Approach

In order to characterize the upregulation of S100P by nitrogen mustard, the following experiments were performed.

In chapter II, a more global picture of this project, what questions were asked, how they were addressed in the experimental approach, and how the focus was narrowed onto S100P are described. In chapter II, the preliminary data of this project is also included. Cell cycle analysis of HL-60 cells and effects on cell growth with HN_2 or DME/DEE treatment can be found in this chapter.

In chapter III, the time course of S100P upregulation by HN_2 in HL-60 cells was tested. The relationship of HN_2 dose and S100P upregulation was also examined. To confirm that the induction of S100P message was also accompanied by an increase in protein, HL-60 cells were treated with HN_2 and cell lysates were immunoblotted for S100P at increasing time points. Subcellular localization of S100P protein was determined by immunocytochemistry microscopy. The time course of S100P message after HN_2 treatment was measured at a series of time points after HN_2 challenge. The HN_2 dose dependency of S100P expression was tested with a series of HN_2 doses. Since it is reported that S100P could be secreted into the cell containing media, S100P protein was quantified in media containing HL-60 cells with and without HN_2 treatment at indicated time points.

In chapter IV, to test whether S100P upregulation after HN_2 treatment is a cell line-specific effect, S100P expression after HN_2 treatment in hematopoietic cell lines U937 and K562, as well as the nonhematopoietic cell lines HBL-100 (human breast cancer epithelial cell line), MCF-10F (non transformed breast cell line) and Colo 320 HSR (colon cancer cell line) were examined. A series of HN_2 dosages near the IC50 (inhibition of 50% cell growth)

(Sunters, Grimaldi et al. 1998) for each cell line was used, and a time course was followed if no difference in S100P expression was detected at varying drug doses. S100P mRNA expression was tested by real time PCR.

In chapter V, to test whether other S100 family members are upregulated by nitrogen mustard as well, expression of other S100 family members was tested after HN_2 exposure in HL-60 cells. S100 family members tested were S100B, which share 50% homology with S100P, S100A8 and S100Z. S100Z is a novel member of S100 calcium binding family that was found to interact with S100P in the yeast two-hybrid system (Gribenko, Hopper et al. 2001). All were tested after HN_2 treatment by real time PCR in HL-60 cells at the indicated time points.

In chapter VI, to determine whether induction of S100P mRNA expression is specific to HN_2 or is a common effect of agents that cause cross-link DNA damage, S100P expression in HL-60 cells was tested after treatment with the cross-linking agents melphalan, MMC, and cisplatin. Melphalan and nitrogen mustard are biochemically similar, and both belong to the mustard family. Both cause cross-links between N7 of two guanines. Mitomycn C also causes cross-links between two DNA strands through a different mechanism. As with other cross-linking agents, cisplatin causes both interstrand and intrastrand cross-links, but intrastrand cross-links are thought to be the main toxic lesion induced by cisplatin.

In chapter VII, cellular response to HN_2 challenge was tested. Cell cycle and cell growth were measured. A karyotype analysis was completed on 20 metaphases from HL-60 cells exposed to HN_2 for 14 d. In this chapter, cells that were exposed to HN_2 before were also rechallenged to test possible development of resistance to HN_2. The relationship

between S100P expression, cell differentiation, cell cycle distribution and cell proliferation was also investigated.

In chapter VIII, the conclusions of the findings of this project are discussed, including the possible role of S100P in response to DNA cross-link damage such as cell survival, cell proliferation, anti-apoptosis, and cell migration and invasion. Future directions include S100 mouse models.

In conclusion, both S100P mRNA and protein are found to be upregulated by DNA cross-linking agents. This upregulation is confirmed with different cell lines and cross-linking agents. These findings suggest a role for S100P in the response of hematopoietic cells to DNA cross-linking agents. Potential specific function of S100P in recognition of DNA damage, cell cycle arrest, apoptosis, DNA repair, or other functions remain to be defined.

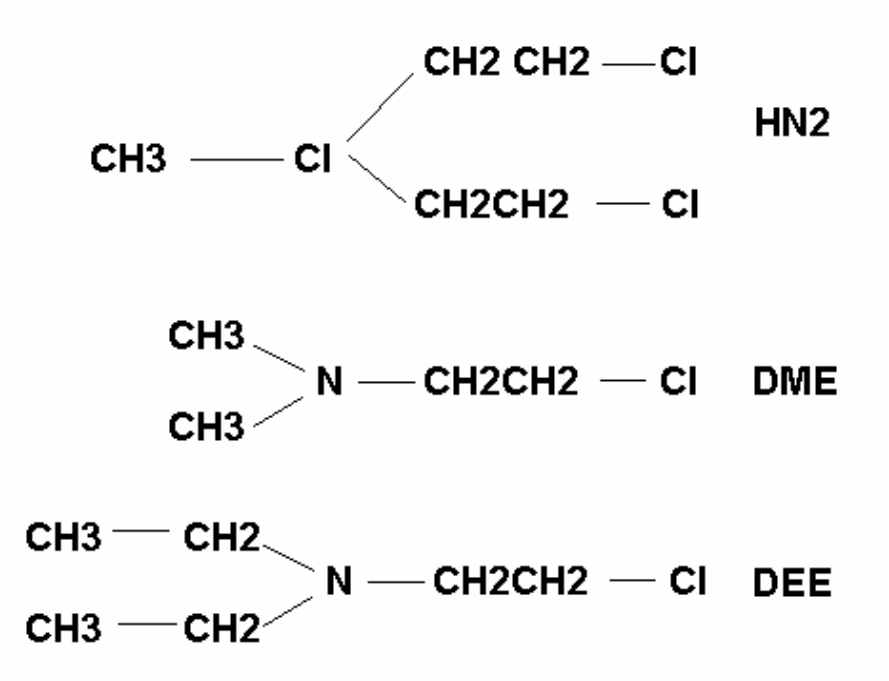


Figure I-1. Structure of bifunctional agent HN_2 and monofunctional agents DME and DEE

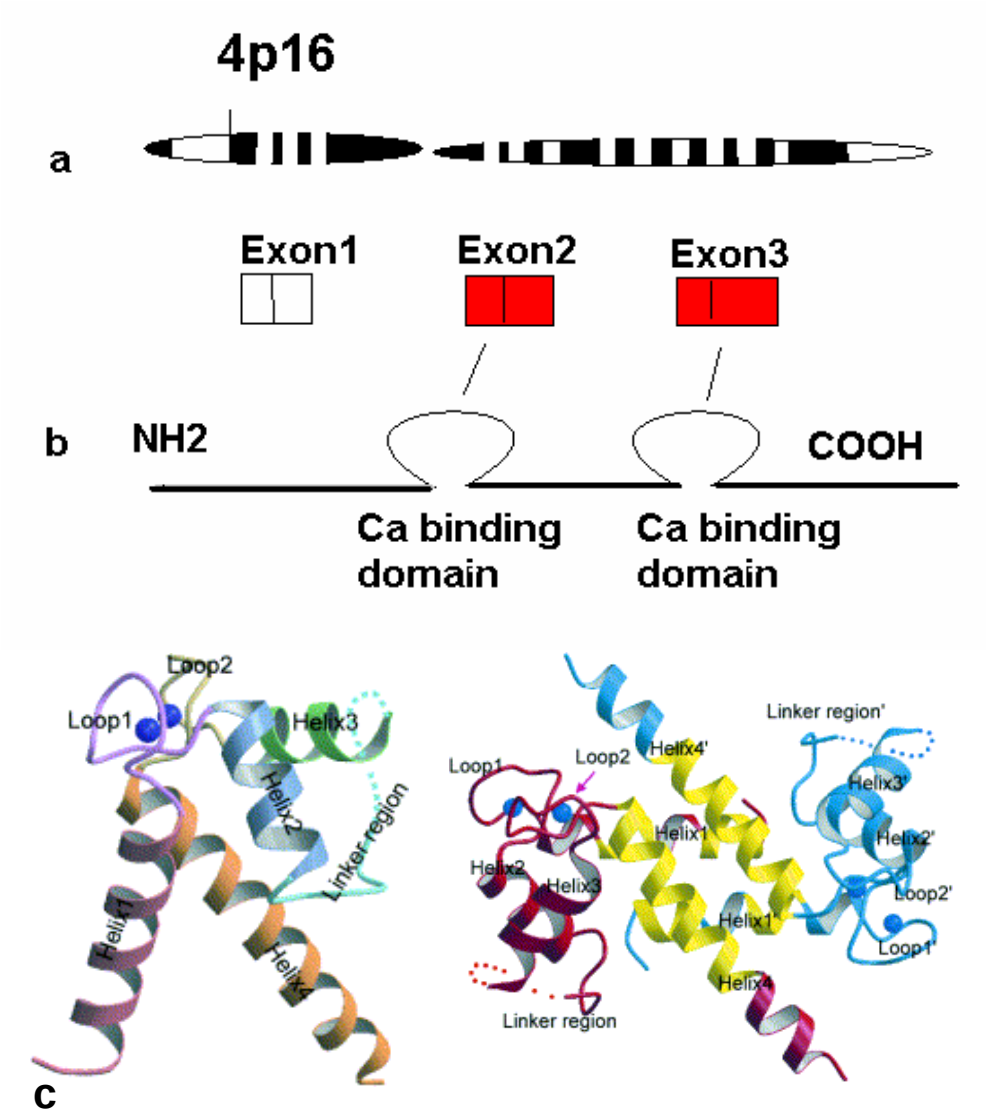


Figure I-2 a. Chromosome location of S100P; b. general structure of S100 calcium binding protein family; c. crystal structure of S100P monomer (left) and dimer (right)(Zhang, Wang et al. 2003).

II.

INITIAL IDENTIFICATION OF DIFFERENTIALLY REGULATED GENES

Introduction

MDS and MDS-related AML are complicated diseases with a multiple step pathogenesis. The molecular pathogenesis of these two diseases remains uncertain. Their diagnosis is based on several factors including clinical history, histology, and cytogenetics, none of which are specific or completely sensitive. The lack of clear understanding of the pathogenesis of these diseases contributes to ineffective therapies and a generally unfavorable prognosis for these patients. Elderly patients are ineligible for bone marrow transplantation, which is the only curative therapy for MDS patients. Unlike chronic myelogenous leukemia and its associated BCR-ABL fusion protein, there are currently no molecular targets for directed treatment of MDS and MDR-AML. There are no animal models or other systemic models for the pathogenesis of MDS and MDR-AML or the preleukemic phenotype of MDS. It has been proposed that MDS results from a combination of increased cell proliferation and increased cell death (Mundle, Iftikhar et al. 1994). However, this hypothesis cannot account for the hypercellular bone marrow in MDS and MDR-AML (Head 1996; Jin, Wang et al. 2003). If there is increased apoptosis, the total number of cells will be decreased in the marrow in a short period of time. In addition, the large amount of cell death should result in increased uric acid, a metabolized product of degradation of purines; the absence of hyperuricemia in these patients is not consistent with an increased amount of cell death.

A multiple step process involving DNA cross-link damage has been proposed for the pathogenesis of MDS and MDR-AML (Head 1996). DNA cross-link damage is implicated in the pathogenesis of MDS and MDS related AML. The HL-60 cell line was used to investigate how DNA cross-link damage may be involved in the pathogenesis of MDS and MDR-AML by searching for genes that respond to cross-link DNA damage. Cells were exposed to the DNA cross-linking agents HN_2, and the gene expression profile was analyzed. Those genes that respond to DNA cross-link damage may be involved in the cellular response to these agents. The cells were also treated with monofunctional alkylating agents (DME and DEE) for comparison, to eliminate the effect of monofunctional alkylation. Dose selection is based on the MiCK (micro culture kinetic) assay (Kravtsov, Greer et al. 1998) and flow cytometry. Maximal concentrations that cause minimal apoptosis were used, which was determined by MiCK and flow cytometry. For HN_2 the dose was 1μM; DME was 50μM; DEE was 100μM; these doses have similar toxic effects on cells. It should be noted that concentrations of DME and DEE equimolar to the HN_2 concentrations used had little toxic effect on HL-60 cells. The doses of DME and DEE that were used are 50 to 100 fold greater that the HN_2 dose to cause the same toxic effect.

HL-60 cells were treated with these agents, and RNA was extracted from each sample at a series of time points after HN_2 challenge. Gene expression profiles were compared among nontreated control, the HN_2 treated group, and the DME/DEE treated group using cDNA macroarray, PCR-select, and cDNA microarray. These three methods are the most commonly used gene expression profile comparison methods; each has advantages and disadvantages. Genes selectively regulated by HN_2 were identified and subjected to further confirmation through virtual northern blot and real time PCR analysis. The expression of these genes was also reproduced in a separate group of experiments.

Multiple analyses identified S100P as a gene consistently and reproducibly upregulated by HN_2.

Materials and Methods

Cell lines. HL-60 (human acute promyelocytic leukemia) cells were obtained from American Type Culture Collection (Rockville, MD). They were cultured in RPMI-1640 medium supplemented with 10% fetal-calf serum (FCS), penicillin, and streptomycin. Cells were maintained in a 37°C, 5% CO_2, fully humidified incubator (using the protocols recommended by the supplier), passed twice weekly, and collected for experimental procedures when in log-phase growth (cell density 4 x 10^5 cells/ml). Viable cells were counted using trypan blue exclusion.

Cross-linking agents and monofunctional alkylating agents. The cross-linking agent analytical-grade mechlorethamine (nitrogen mustard, HN_2), and the monofunctional alkylating agents (2-Dimethylaminoethyl chloride (DME) and 2-Diethylaminoethyl chloride (DEE)) were purchased from Sigma-Aldrich (St. Louis, MO USA).

Cell cycle analysis. 2×10^6 cells with or without challenge of alkylating agents were collected and fixed in 5 ml ice-cold 70% ethanol, and stored at -20°C overnight in the dark. The ethanol was removed by washing with PBS, and the cells were incubated in 500 µl propidium iodide (0.1 mM EDTA, 0.02 mg/ml propidium iodide, 0.1% Triton X-100 in PBS; Sigma) for 30 minutes at room temperature in the dark. Flow cytometric analysis of DNA content was then performed using a FACScan analyzer (Becton Dickinson, Mountain View, CA). Cell cycle analysis was conducted using ModFit cell cycle software and WinList histogram software (Verity Software House, Topsham, ME). Apoptotic cell death was

quantified as the proportion of cells in the population with a subdiploid (<2N) DNA content.

Microculture kinetic (MiCK) assay. The MiCK assay is an automated optical density based assay that detects membrane blebbing at 5 min intervals over 72 hours. The MiCK assay provided both the time course and the extent of apoptosis initiated by alkylating agents. The assays were completed as previously described (Kravtsov, Greer et al. 1998). Cells were suspended in complete medium at 2 x 10^5 cells/ml, plated in 240-μl aliquots in a 96-well micro titer plate (Corning-Costar, Cambridge, MA), and incubated in a fully humidified atmosphere of 5% CO_2 for 60 minutes. Appropriate dilutions of HN_2 were added to wells in 10-μl aliquots to achieve final concentrations of 0.2, 0.4, 0.6, 0.8, 1, 1.25, 1.5 and 2 μmol/L. After incubation at 37°C for 30 minutes in a completely humidified atmosphere of 5% CO_2 in air, 50 μl of sterile mineral oil (Sigma, St. Louis, MO) were layered on the top of each microculture. The microtiter plate was then placed in the incubation chamber of a spectrophotometer (SPECTRAmax 340, Molecular Devices Corp., Sunnyvale, CA), incubated at 37°C, and the OD at 600 nm was read every 5 minutes for a period of 72 hours. The reader was calibrated to zero absorbance using wells containing only complete medium without cells. The extent of apoptosis was expressed as both kinetic units (KU) of apoptosis and percentage of apoptotic cells using methods described in the literature (Kravtsov, Greer et al. 1998).

RNA extraction. Cells in log-phase growth with and without HN_2 challenge, were harvested from 5 ml cultures. Total RNA was prepared using RNeasy® Mini Kits (Qiagen Inc. Valencia, CA) and used for virtual Northern blot and real time quantitative PCR.

cDNA microarray. Total RNA isolated from untreated cells or those exposed to HN_2, DEE or DME for 1, 6, 24 or 30 hours. was used as template for labeling with cy3 or

cy5 and then hybridized to a human 11K cDNA microarray (Research Genetics) by the Vanderbilt Microarray Shared Resource (Vanderbilt University). GenePix Pro (Axon Instruments, Inc.) was used to scan microarrays and perform basic analysis of arrays and generating lists of differentially expressed genes. The microarray results and a description of the experiments in MIAME format are available at: www.vmsr.net/supl/.

Virtual Northern blot. A probe of the S100P gene was hybridized to a blot containing cDNAs proportionally amplified from total cellular RNA. Full-length first strand cDNAs were generated from total RNAs of control and drug treated cells using SMART™ PCR cDNA synthesis kit (BD Biosciences Clontech, Palo Alto, CA). Double stranded cDNA was then amplified by long distance PCR (Clontech, Palo Alto, CA), electrophoresed on a 1.2% agarose gel, denatured and transferred onto Hybond N+ Membrane (Amersham, Arlington Heights, IL). A clone of the S100P cDNA spotted on the microarray was obtained from The Vanderbilt Microarray Core. Plasmid DNA was purified using Promega wizard plasmid purification kit at mini-scale and partially sequenced to confirm identity. The probe was excised by restriction enzyme digestion, labeled with fluorescein using ECL random primer labeling (Amersham, Arlington Heights, IL) and hybridized with membranes in Quickhyb (Stratagene, La Jolla, CA) at 60 °C for 2 hours, and washed once with 1xSSC, 0.1%SDS; 0.5xSSC, 0.1%SDS; 0.1xSSC, 0.1%SDS for 15 minutes each at 60 °C. Detection was performed by the ECL luminescence detection method (Amersham, Arlington Heights, IL). A probe for G3PDH was used to normalize cDNA loading.

Real time PCR. Total RNA (500 ng) was used to prepare cDNA with SuperScript™ cDNA synthesis kit and oligo dT (Invitrogen, Carlsbad, CA USA). The TaqMan® gene specific expression assay for S100P was obtained from Applied Biosystems (Foster City, CA USA) and reactions performed in triplicate using an ABI PRISM 7700

Sequence Detection System (Applied Biosystems). Ribosomal 18S RNA was used as an internal reference standard and relative quantitation of S100P mRNA expression was analyzed using SDS Version 1.7 software (Applied Biosystems). The relative Ct (threshold cycle number) corrected for the internal reference standard with standard deviation and coefficient of variation (CV) was calculated as described in ABI protocols (Applied Biosystems). The relative quantitation value of S100P, normalized to endogenous control 18S rRNA and relative to a calibrator, is expressed as $2^{-\ Ct}$ (fold), where Ct=Ct of target gene (S100P)-Ct of endogenous control gene (18S rRNA), and Ct= Ct of samples for target gene- Ct of the calibrator for the target gene. In each case, real time PCR was performed three times with different RNA preparations.

Results

Cellular effects of alkylating agents. HL-60 cells were treated with HN_2, DME and DEE at the maximum tolerated dose that did not induce significant apoptotic cell death (defined as >5% cell death, determined by flow cytometry and MiCK assay analysis). For HN_2, the relevant dose was 1 μM, and for DME and DEE the dosages were 50 and 100 μM respectively (Figure II-1).

At 24 hours after addition of 1 μM HN_2, an obvious late S/G2 arrest was detected by flow cytometric analysis of DNA content (Figure II-2). Over 96 hours at time points of 48, 72 and 96 h, the cell cycle DNA distribution returned to a similar distribution to untreated cells (Figure II-2).

Interestingly, cells arrest at different stages with bifunctional and monofuctional drug treatment. HN_2 causes late S/G2 arrest (Figure II-2) while DME and DEE cause early S phase arrest (Figure II-3).

S100P mRNA is increased in HN_2 treated HL-60 cells relative to untreated and monofunctional agent-treated cells. Microarray gene expression profile analysis revealed a 4-fold increase of S100P expression in 1μM HN_2 treated HL-60 cells at 24 hours relative to an untreated and DME (50 μM) or DEE (100 μM) treated cells (Figure II-4). This upregulation was confirmed by virtual Northern blot analysis (Figure II-5), and real time PCR analysis (Figure II-6). cDNA microarray consistently suggested S100P overexpression in HN2 treated cells relative to DME and DEE treated cells at 1, 6 and 30 hours (Table 1).

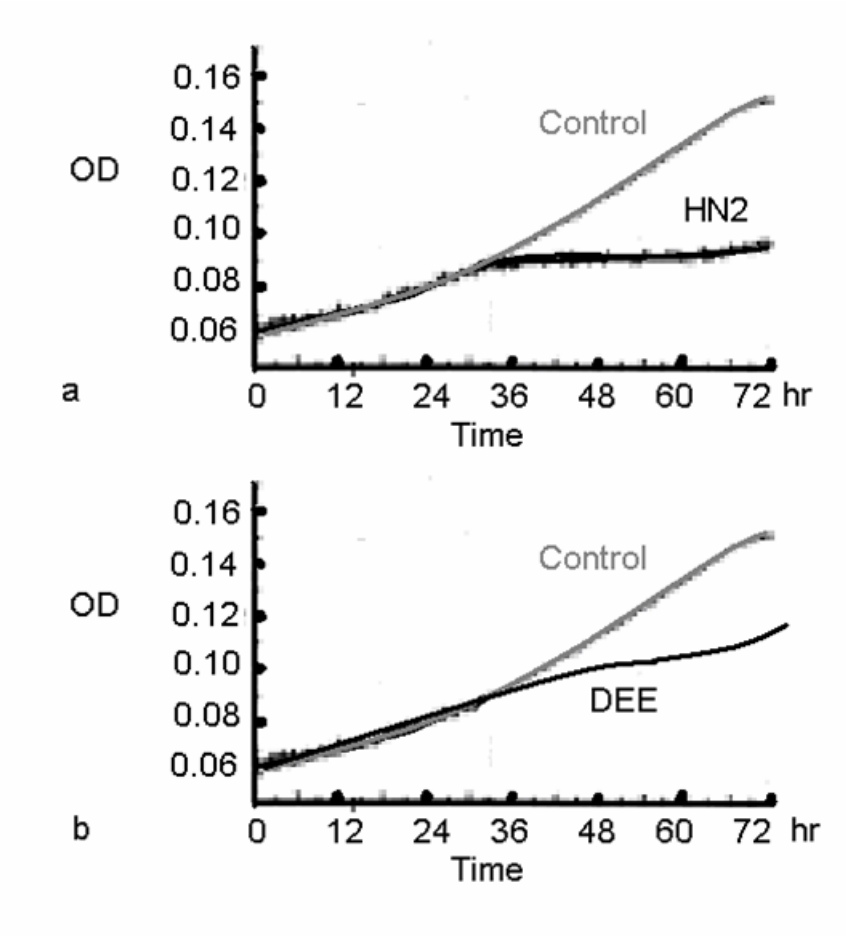


Figure II-1. Concentrations of HN_2 (1μM) and DEE (100μM) that cause minimal cell death in HL-60 cells determined by MiCK assay.

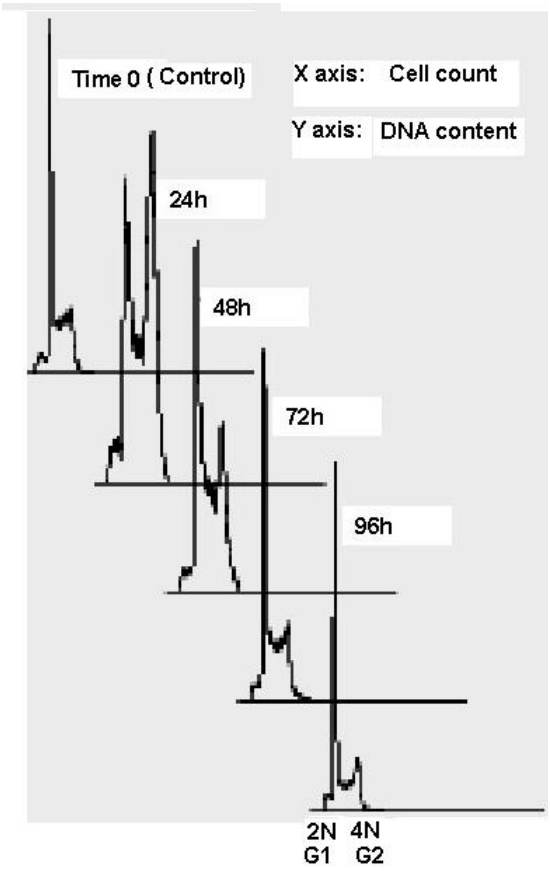


Figure II-2. Effects of 1.0 μM HN_2 on HL-60 cell cycle distribution. HL-60 cells were treated with HN_2 over a 96 hours period. The cell cycle distribution was assessed by quantitation of cellular DNA analyzed by flow cytometry.

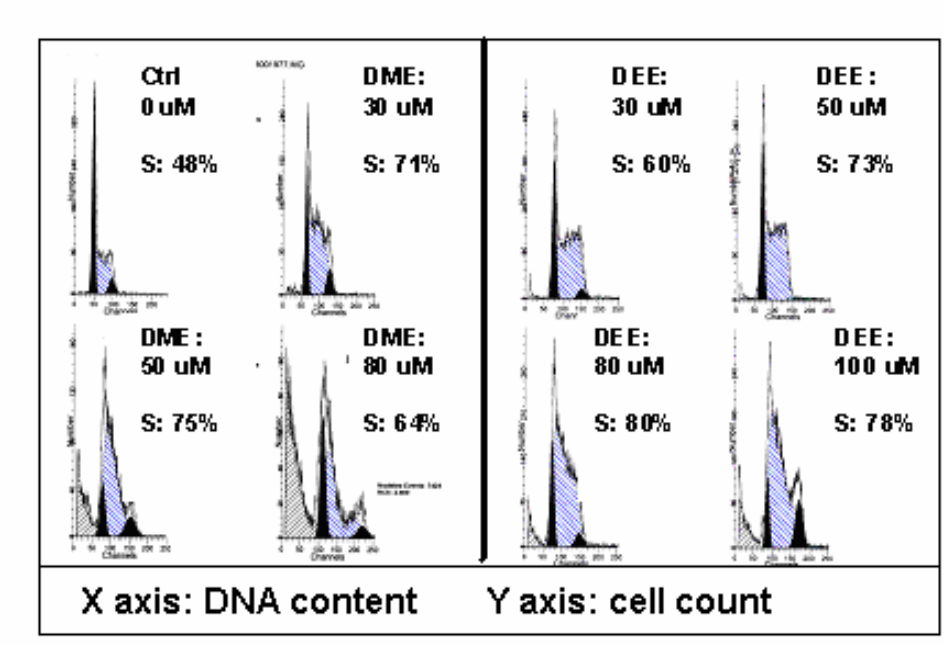


Figure II-3. S phase Cell cycle arrest in HL-60 cells induced by monofunctional agents DME and DEE at 24 hours, measured by flow cytometry.

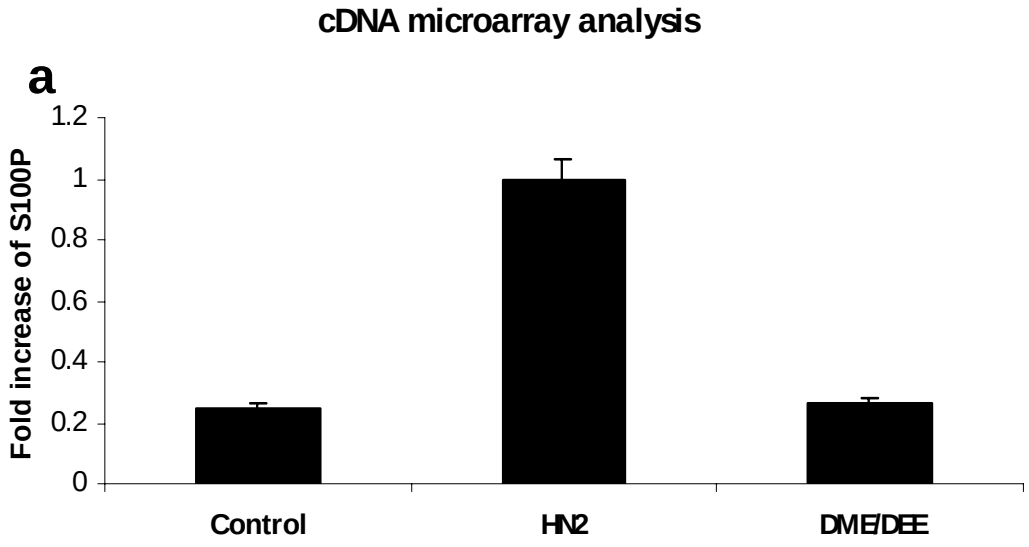


Figure II-4. Upregulation of S100P mRNA by HN_2, but not by monofunctional alkylating agents DME / DEE. Total RNA was harvested from untreated cells and those treated with 1 μM HN_2, 50 μM DME and 100 μM DEE 24 hours after treatment. S100P mRNA level in each sample was measured by cDNA microarray analysis. Y axis: fold increase of S100P over control. Error bar; standard deviation.

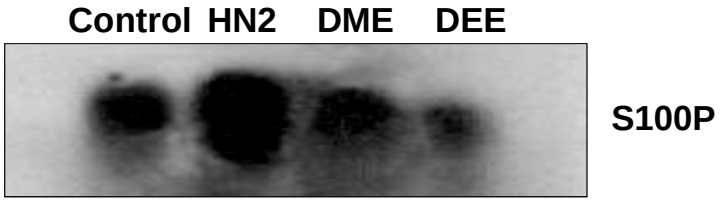


Figure II-5. Virtual Northern analysis of induced S100P mRNA by HN_2 and not by monofunctional alkylating agents DME or DEE. The cDNA was proportionally amplified from total cellular RNA isolated from cells untreated and those treated with 1μM HN_2, 50μM DME or 100μM DEE for 24 h. Sample loading was adjusted for equal G3PDH signal intensity. S100P was specifically upregulated by HN_2.

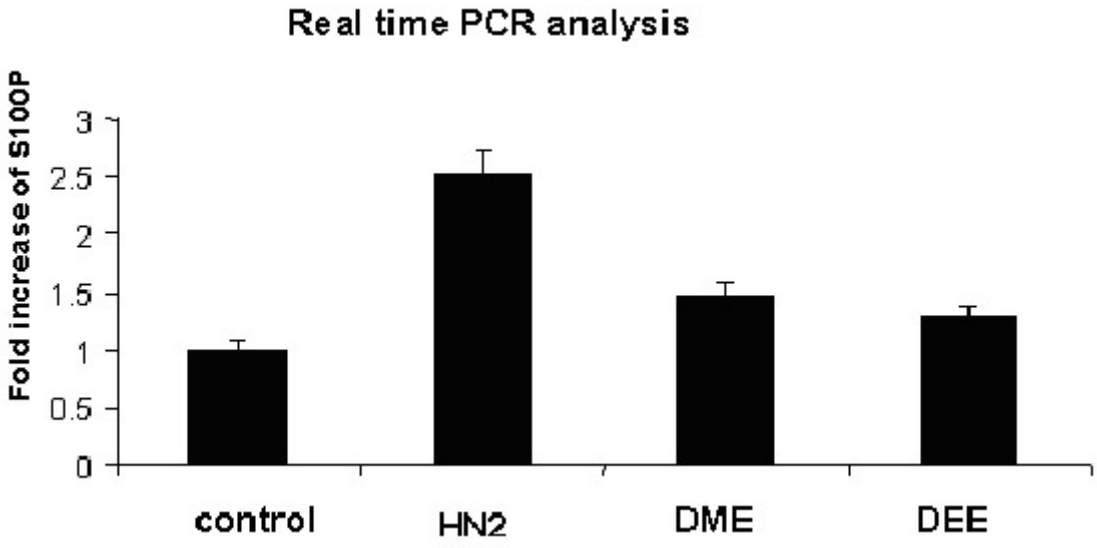


Figure II-6. Real time PCR analysis of S100P in HL-60 cells treated with 1 μM HN_2, 50 μM DME, 100 μM DEE and untreated control at 24 hours. The error bars are CVs that were consistently less than 5%.

Table 1. Specificity of HN_2 versus monofunctional alkylating drugs (at 1, 6, and 30 hours after treatment) to induce S100P expression as observed in the analysis of transcript levels by cDNA microarray. "-": not determined

S100P relative expression levels from microarrays

Arrays	1	6	30 (h)
HN_2/DME&DEE	2.2X	2.9X	3.5X
HN_2/NT *	-	-	4.1X

* NT = Not treated

Discussion

The pathogenesis of MDS and MDS-related AML are not understood. There is an increased incidence of MDS and MDR-AML in Fanconi Anemia patients and in patients who undergo chemotherapy with alkylating agents and agents that cause cross-link DNA damage. Fanconi Anemia patients have increased sensitivity to DNA cross-linking agents. These observations suggest that interstrand cross-link DNA damage plays a role in the pathogenesis of MDS and MDR-AML.

To mimic the cellular response to DNA cross-linking damage, the tumor cell line HL-60 as treated with the cross-linking agent nitrogen mustard and analyzed gene expression profile changes. Two monofunctional alkylating agents (DME and DEE) were used for comparison. This strategy was employed to identify HN_2-selectively-regulated genes. A series of HN_2 concentrations were used to identify the optimal dose to use for the microarray experiment. It was found that 1 μM HN_2 caused a minimal amount of apoptosis in HL-60 cells. At concentrations of 1 μM and lower, HN_2 causes G2 arrest in HL-60 cells. Similarly toxic doses for DME and DEE were also used, which are 50 μM and 100μM respectively. It should be noted that 1 μM HN_2 is a relatively high concentration since it can abrogate the PHA mediated stimulation of PBMCS culture (data not shown). The difference in tolerance may be because HL-60 is a tumor cell line, while PBMCS are normal cells. The lack of functional p53 in HL-60 cells may lead to increased drug tolerance in these cells, resulting in the need for higher doses of drugs to cause cell cycle arrest following DNA damage.

Another interesting observation is that HN_2 causes late S/G2 cell cycle arrest, while the monofunctional agents DME/DEE cause earlier S phase arrest. The explanation may be that the DNA interstrand cross-links formed between two DNA strands block the

completion of DNA replication and mitosis. In addition, the concentration of DME and DEE used was 50 to 100 times higher than HN_2. It may take relative large amount of monoadducts to cause similar toxic effects versus DNA cross-linking damage.

To make the comparison between the HN_2 treated, control, and monofunctional agents treated groups, several methods were tried before cDNA microarray analysis was used. These included cDNA macroarray and PCR-select. A macroarray plate carries much fewer genes than cDNA microarray, which limits its power in analysis to find candidate genes that respond to HN_2. PCR-select is labor intensive, takes a long time to finish one cycle from extracting RNA to obtain a list of candidate genes, and has a high false positive rate making reproducibility difficult. Technically, these two methods are time consuming and costly.

Microarray analysis provides information about a large number of genes and allows detailed statistical analysis for gene selection. It takes a shorter period of time for each cycle of search. The combination of these features allowed the development of a list of genes that had a high probability of being specifically regulated by HN_2. A disadvantage of this approach for this experiment is the disparity of labeling efficiency. For example, when comparison between the control and HN_2 treated HL-60 cells was made, the RNA from the latter group is not as intact as the control group RNA. In addition, HN_2 can interact with RNA. This results in different labeling of sample and control with cy5 and cy3 in the microarray analysis. To avoid this problem, for each group of experiments reverse labeling and comparison (A vs B and B vs A) was done. Only those genes that consistently appeared in those two comparisons were considered as candidate genes. These genes may have potential diagnostic value in the future and also may provide targets for additional

investigations into the cellular mechanisms of MDS and MDR-AML. This research was focused on genes that were reproducibly altered by HN_2.

S100P was found to be selectively upregulated by HN_2 compared to control and monofunctional agent-treated groups. Its upregulation was reproducible and confirmed through several methods. The upregulation of S100P after HN_2 exposure suggests that it plays a role in the response to DNA cross-link damage. S100P is one of the least studied genes of the S100 calcium binding protein family. Current publications link its overexpression to tumor initiation and progression (Amler, Agus et al. 2000; Guerreiro Da Silva, Hu et al. 2000). Along with other S100 proteins, S100P is exclusively expressed in vertebrates (Kretsinger 1976). Selective high expression of S100P in bone marrow, the calcium binding property of S100P, and evolution of S100P in vertebrates suggest S100P expression may have evolved in parallel with the migration of hematopoiesis to its intramedullary location. The previous findings plus the upregulation of S100P by HN_2 and other cross-linking agents in marrow cell lines suggest parallels to the Fanconi protein system, a system that is also exclusively expressed in vertebrates and is involved in the cellular response to cross-link DNA damage. The FA protein system also serves an unknown but critical protective role in marrow and bone as evidenced by bone and bone marrow abnormalities in FA.

Other interesting genes were identified, but for most regulation by HN_2 was difficult to reproduce. It is possible that the asynchronous culture of HL-60 cells was a problem. During the experiment, the cells that are exposed to the cross-linking agents are at different points in the cell cycle. This may result in different gene expression response patterns in different cells during each experiment. One way to avoid this would be to synchronize the cells. Another way is to sort different cell cycle populations and compare cells from

different samples that are at the same cell cycle stage, or compare different cell cycle stages, in the same sample. For example, the G2 population of non-treated HL-60 cells and HN_2 treated HL-60 cells at 24 hours after treatment could be sorted, and a microarray to compare gene expression patterns could be performed. Alternatively, elutriation has been used to isolate cells in specific phases of the cell cycle. However, since S100P was a gene of interest, the alternatives were not pursued.

With these findings, it was asked, is this S100P response unique to the HL-60 cell line? Is it unique to HN_2? Do other cross-linking agents upregulate S100P? Is it unique to S100P? Do other S100-calcium binding family members respond the same way to drug challenge? Does the S100P protein expression respond to HN2 treatment? Where is the S100P protein localized, and is it secreted? In the next few chapters, these questions were addressed.

III.

CORRELATION BETWEEN INDUCTION OF mRNA AND INDUCTION OF S100P PROTEIN

Introduction

S100P mRNA is selectively upregulated by 1 μM nitrogen mustard (HN_2) at 24 hours after drug challenge. In order to observe the kinetics of the upregulation, S100P expression at multiple time points after HN_2 exposure by real time PCR amplification was tested. The effect of varying doses of HN_2 on the expression of S100P was also examined.

In the preliminary data, the upregulation of S100P mRNA level by HN_2 was identified through microarray analysis, and confirmed by virtual northern blots and real time PCR data. Because RNA expression may not correlate with protein expression, to evaluate the S100P protein response to HN_2, we tested S100P protein expresssion by Western blot at series of time points. To explore the possible physiological role of S100P, the cell cycle distribution of S100P protein expression was analyzed by flow cytometry, quantitating S100P protein versus DNA content. This was used to determine S100P protein intracellular levels, confirming Western blot data.

The subcellular localization of S100P protein in hematopoietic cell lines has not been previously described and may provide insight to the function of S100P and its role in the response to DNA cross-link damage. Immunocytochemistry (ICC) was used to detect the localization of S100P protein in HL-60 cells and to observe cellular levels of S100P over time. Possible changes in S100P subcellular location of S100P after HN_2 challenge was also examined by immunocytochemistry. Some proteins in the S100 family are secreted by cells.

It has also been reported that S100P is secreted into media when expression is transgenically induced in NIH3T3 cells, which do not otherwise express S100P (Arumugam, Simeone et al. 2004). It is unknown whether S100P is secreted into media by HL-60 cells. If it is, it will be important to know if this secretion is regulated by HN_2 in HL-60.cells, which do express S100P, in the presence or absence of HN_2 treatment. Therefore, we also analyzed HL-60 containing media for evidence of S100P secretion.

Materials and Methods

Cell lines. As described previously.

Cross-linking agents and monofunctional alkylating agents. As described previously.

RNA extraction. As described previously.

Real time PCR. As described previously.

Protein extraction. HL-60 cells were treated with 1 μM HN_2, and proteins were extracted at specified time points. The cells were washed in phosphate-buffered saline (PBS) and lysed in sample buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol) (Sigma), and then homogenized with a polytron homogenizer (6000 rpm) with a protease inhibitor cocktail (Complete TM) (Roche, Nutley, NJ). Protein extracts were centrifuged for 15 min at 4 °C at 12,000 rpm in an Eppendorf centrifuge, and the supernatants were used for Western blot analysis. Protein concentration was estimated by the Bradford procedure (Bio-Rad, Hercules , CA) with bovine serum albumin as a standard.

Antibodies. Anti-S100P primary monoclonal mouse antibody was purchased from BD Biosciences (Transduction Laboratories, Lexington, KY).

Western blot. Whole cell protein extracts (25 μg) were electrophoretically separated on 15% SDS gels and transferred to PVDF membranes (Millipore Corporation, Billerica, MA). The blot was blocked for non-specific protein binding and probed with an anti-S100P primary monoclonal mouse antibody (1:100) (BD Transduction Laboratories, Lexington, KY USA). Antibody binding was detected using an HRP-conjugated goat anti-mouse IgG (1:4000)(Jackson Immuno Research Lab. Inc., West Grove PA USA). The antibody interaction was visualized by ECL detection (Amersham, San Francisco, CA, USA). Human placental lysates were used as a positive control (Transduction Laboratories, Lexington, KY USA).

Immunocytochemistry. Slides of HL-60 cells were prepared by cytocentrifugation. After air drying cells were fixed and endogenous peroxidase activity quenched in methanol with 3% H2O2 at room temperature for 10 min. Cells were hydrated through gradient alcohols to phosphate buffered saline (PBS). Nonspecific protein binding sites were blocked with 1% bovine serum albumin, 10% bovine calf serum in PBS for 30 min. The slides were exposed to a monoclonal anti-S100P antibody (Transduction Laboratories) diluted 1:15 in blocking solution for 18 h. at room temperature. An avidin-biotin-peroxidase complex (Vector Laboratories, Inc., Burlingame, CA) based detection system was used to visualize the antibody interaction. Reagent control slides were included in each experiment as well as an isotype control antibody.

Quantitation of S100P protein by flow cytometry. Cellular S100P content was measured in HL-60 cells from 0 to 96 hours after 1 μM HN_2 treatment. Cells were washed twice in PBS +2% FCS and fixed in 70% EtOH in the dark at room temperature for 1 hour. Cells were then washed twice in cold 1X PBS. A saturating amount (as determined by titration on HL-60 cells) of S100P antibody was added and allowed to incubate in the dark at

room temp for 1 hour. A matching concentration IgG1-Isotype tube was also prepared in the same fashion. Cells were washed twice in PBS + 2% FCS. A FITC-labeled IgG1-goat anti-mouse antibody at 1 μg/μl was added to S100P and isotype tubes and incubated for 1 hour. Cellular DNA was stained with 7-AAD. An FC500 cytometer (Beckman-Coulter) with a 488 nm and 633 nm excitation source was used for data acquisition. S100P data were analyzed using WinList 5.0, ModFit LT 5.0 (Verity Software House, Topsham, ME) and Excel 5.0 (Microsoft, Seattle WA). S100P expression was determined by comparison of each time point to the background protein expression as determined by the isotype control in drug treated and control samples. This method was also used to measure the frequency and intensity of S100P expression in each cell cycle state. Cell cycle data was determined by modeling in ModFit LT and analyzed using Lysys software (Becton Dickinson).

Quantitation of MPM2 protein by flow cytometry. Please refer the above paragraph for details of the methods. MPM2 antibody was provided from Esoterix (Brentwood, TN).

ELISA for S100P. S100P was quantified in the media collected from 1 μM HN_2 treated cells at specified time points after HN_2 treatment. The media was collected and concentrated using YM10 centricon concentrating filters. (Millipore, Billerica, Mass.,USA,) Concentrated samples (100 μl) were incubated for 2 hours at room temperature in antibody coated plates and washed thrice with wash buffer. S100P was captured between an anti-bovine S100 calcium-binding protein B (S100B) rabbit polyclonal antibody (Abcam Ltd.,Cambridge, UK) and an S100P specific monoclonal antibody, using an ELISA kit (Protein Detector ELISA kit, KPL, Gaithersburg, Maryland, USA). Bound S100P was detected with a horseradish peroxidase (HRP) labeled anti-rabbit secondary antibody and using tetramethylbenzidine (TMB) substrate. Color development was blocked with 1M

phosphoric acid and read at 450nm. S100P in a placental lysate was used as a positive control, and media with or without serum were used as negative controls.

Results

Time course of S100P mRNA induction by HN_2. HL-60 cells were treated with 1 μM HN_2, total RNA was extracted after 1, 2, 6, 24, 48, and 72 hours, and relative quantification of RNA by real-time PCR was performed. Human placental RNA was used as a positive control for S100P amplification. S100P mRNA was not measurably upregulated at early time points (1, 2, and 6 hours after treatment), but increased 2.3 fold over baseline at 24 hours, 8.3 fold at 48 hours and 76.4-fold at 72 hours after HN_2 challenge (Figure III-1).

The increase in S100P mRNA levels is HN_2 dose-dependent. To determine if the observed increase in S100P mRNA levels is HN_2 dose-dependent, HL-60 cells were treated with 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, and 5 μM HN_2, and total RNA extracted 24 hours after treatment. Real-time PCR revealed that S100P mRNA was progressively upregulated from 2-fold at 0.25 μM to 10-fold at 2.5 μM over baseline (Figure III-2). At 3 and 4 μM HN_2, the expression level of S100P drops; at these doses, there is a significant amount of cell death. The HL-60 cell culture was almost destroyed by 5 μM HN_2.

S100P protein levels are upregulated with HN_2 treatment. To confirm that the induction of S100P message was also accompanied by an increase in protein, HL-60 cells were treated with 1μM HN_2 and cell lysates were immunoblotted for S100P at the indicated time points (Figure III-3). Placental lysates were used as a positive control. S100P protein levels in HL-60 cells are undetectable in untreated cells as well as cells at 2, 24, (not shown)

45 and 70 hours after treatment. S100P protein was strongly induced at 90 hours post treatment with HN_2 (Figure III-3 A).

Flow cytometry for S100P and cell cycle analysis. A subpopulation of cells with G2/4N DNA content expressed a high level of S100P at 96 hours after HN_2 (Figure III-3 B). An increasing number of cells with elevated S100P were observed at 96 hours with increasing levels of HN_2 (Figure III-3C). The elevated level of S100P expression detected by flow cytometry in a subset of cells at 96 hours (Figure III-3B and C) appeared to correlate with immunoblot detection of S100P at approximately the same time after adding HN_2 (Figure III-3A).

S100P protein subcellular localization. Immunoperoxidase localization of S100P in untreated HL-60 cells showed an occasional positive cell with low level of expression within cytoplasm only (Figure III-4A). Immunoperoxidase analysis of S100P at 24 hours after HN_2 showed a few weak positive staining cells (Figure III-4B). An increased number of more reactive cells (Figure III-4 C) were detected at 96 hours after HN_2. These reactive cells appear to coincide with the detection of a subset of G2/4N cells with high level of immunoreactivity by flow cytometry (Figure III-4B and C). At 192 hours after HN_2, a large number of reactive cells were detected (Figure III-4D).

The immunocytochemical analyses consistently showed cytoplasmic reactivity only, indicating that HN_2 did not alter S100P intracellular localization. In addition, mitotic cells in cultures untreated or treated with HN_2 showed a low level or no S100P immunoreactivity.

HL-60 cells treated with DME (50 μM) for 96 hours showed the same rare weak S100P reactive cells as with untreated cells (data not shown). The results were consistent with the specificity of HN_2 versus monofunctional alkylating drug to induce S100P mRNA upregulation.

S100P is not secreted by HL-60 cells before or after HN_2 treatment. A sandwich ELISA was used to examine the extracellular S100P levels in HL-60 cell cultures. No detectable levels of S100P were observed in medium from HL-60 cells untreated or treated with HN_2 for 96 hours (data not shown). Placental lysate containing S100P was used as a positive control. The baseline level of S100P in the media containing HL-60 cells without drug treatment was very close to negative control levels. S100P levels in the media at 24, 48, 72, and 96 hours after HN_2 treatment did not change significantly, indicating that a measurable amount of S100P protein is not secreted by HL-60 cells into media before or after HN_2 treatment.

Discussion

S100P mRNA was found to be selectively upregulated by HN_2 but not by monofunctional alkylating agents. To characterize these findings, the kinetics of S100P upregulation by HN_2 was analyzed. At 1 μM HN_2, the upregulation of S100P mRNA is continuous during the time points assayed. The mRNA increase starts at 24 hours and increases dramatically by 72 hours. These findings suggest S100P upregulation is not transient. The underlying mechanism remains uncertain.

S100P upregulation is HN_2 dose dependent. DNA damage increases with increasing HN_2 doses and thus the dose dependency of S100P suggests it may have a role in response to DNA damage, but its potential role is unclear.

S100P protein increases also, but is delayed versus the mRNA response. In HL-60 cells with no drug challenge, S100P protein was hardly detectable by Western blot, and no change was detected in protein expression up to 72 hours, but S100P was strongly induced by 90 hours after 1 μM HN_2 treatment. S100P mRNA expression is tissue specific (Jin, Wang et al. 2003), and S100P mRNA level is very high in HL-60 cells compared to other tissue and cell lines. However, S100P protein is expressed only at very low levels at baseline, and there is a lag between mRNA overexpression and S100P protein increase. Flow cytometry also confirmed the upregulation of S100P protein; and interestingly, a subpopulation of cells in G2 expresses very high levels of S100P protein after treatment.

Immunocytochemistry revealed at 96 hours, a group of cells with strong cytoplasmic staining of S100P (Figure III-4); this subset of cells has a unique morphology type. S100P appeared to localize in the cytoplasm of HL-60 cells. These cells were larger than most cells, with kidney bean shape nuclei, and with a big negative staining cytoplasm area adjacent

to nuclei, and appearing to represent the Golgi apparatus. These cells may correspond to the S100P protein detection in the Western blot. Characterizing this group of cells may help to illuminate the function of S100P. Cell cycle arrest in G2 occurs around 24 hours; is largely gone by 48 hours, and at 96 hours, the cell cycle distribution of HL-60 cells is similar again to normal cells (Figure II-2).

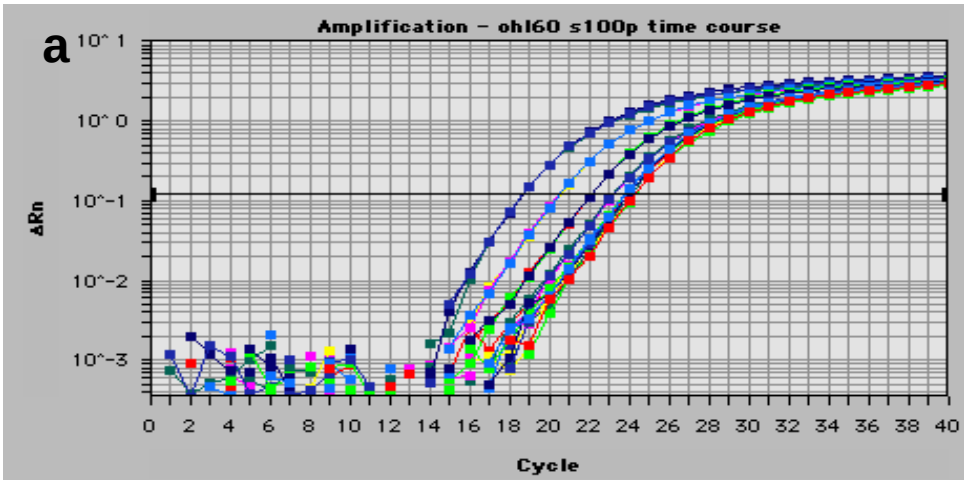
S100P protein overexpression occurs at 96 hours, as opposed to G2 cell cycle arrest at 24 hours. There is no significant subG1 population throughout the first four days of the time course. Given these findings, S100P is unlikely to be directly involved in the G2 cell cycle arrest or cell death. The possible role of the upregulation at later time points includes promotion of cell survival through overcoming G2 cell cycle arrest. The regulation of S100P protein with the cell cycle suggests a G2-M transition function, but underlying mechanism remains unclear. More discussion on this topic is found in Chapter VII.

The subcellular localization of S100P in the cytoplasm in HL-60 cells is reported for the first time here. No obvious relocalization was found in HN_2 treated HL-60 cells versus resting cells. Cytoplasmic localization may provide clues to the function of S100P. For example, the non-nuclear localization of S100P indicates that it does not have a direct role as a transcriptional regulatory factor, a direct role in DNA repair, or recognition of DNA damage.

Some members of the S100 calcium-binding protein family are secreted into the media (Donato 2001). It was recently reported that S100P is secreted into media by S100P transfected NIH3T3 cells (Arumugam, Simeone et al. 2003). We were unable to demonstrate secretion of S100P protein in HL-60 cell containing media, either with or without HN_2 treatment. The difference in cell types may account for these different results; HL-60 cells are a human hematopoietic tumor cell line, while NIH3T3 cells are non-

transformed mouse fibroblast cells. In addition, while HL-60 cells express S100P normally, it is not normally expressed in NIH3T3 cells. It is possible that the overexpression of S100P in a normally non-expressing cell line may have resulted in non-physiologic secretion into media. It should also be noted that S100P does not contain a classic signal peptide for secretion. Lack of signal peptide may suggest S100P is not secretive protein although many members of S100 family are secreted without signal peptide. Also, ICC showed no apparent localization of S100P protein in Golgi apparatus, given the empty paranuclear area without S100P staining in the cytoplasm in HL-60 cells, while many secreted proteins are localized in Golgi before they are secreted. .

In summary, upregulation by HN_2 is both time and dose dependent. Both S100P mRNA and protein levels are overexpressed after drug challenge wth a lag in protein versus mRNA expression. After HN_2 exposure, S100P protein expression is high in a subset of G2 cells, suggesting cell cycle regulatory role for S100P. S100P protein secretion is not detected with and without HN_2 treatment by "sandwich ELISA".



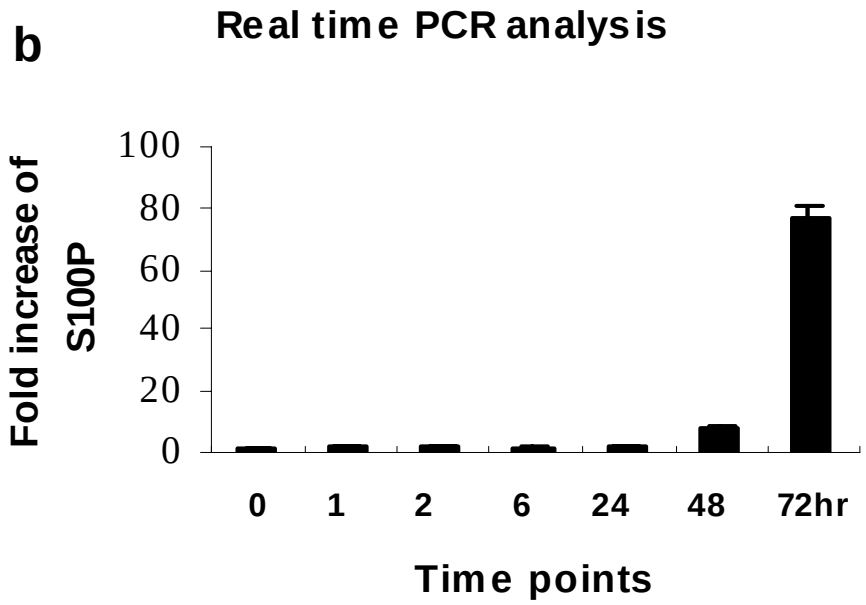


Figure III-1. Upregulation of S100P mRNA by HN_2 in HL-60 cells is time dependent. a. Raw data Real time PCR quantitation of S100P time course. Total RNA was isolated at serial time points (0, 1, 2, 6, 24, 48, 72 hours) after 1 μM HN_2 challenge from HL-60 cells, and used for real time PCR amplification. Each plot represents an amplification curve. ΔRn: Normalized reporter signal. b. Data analysis of real time PCR. The error bars are CVs that were consistently less than 5%.

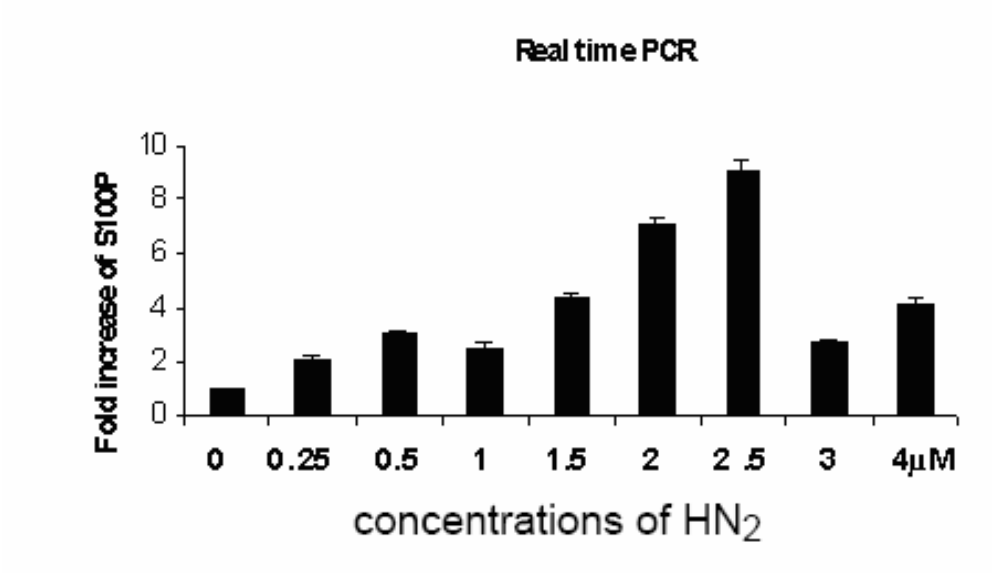


Figure III-2. Upregulation of S100P mRNA by HN_2 in HL-60 cells is dose dependent at 24 hours. Total RNA isolated from HL-60 cells after challenge with serial HN_2 doses (0, 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4 μM) was used for real time PCR amplification. The error bars are CVs that were consistently less than 5%.

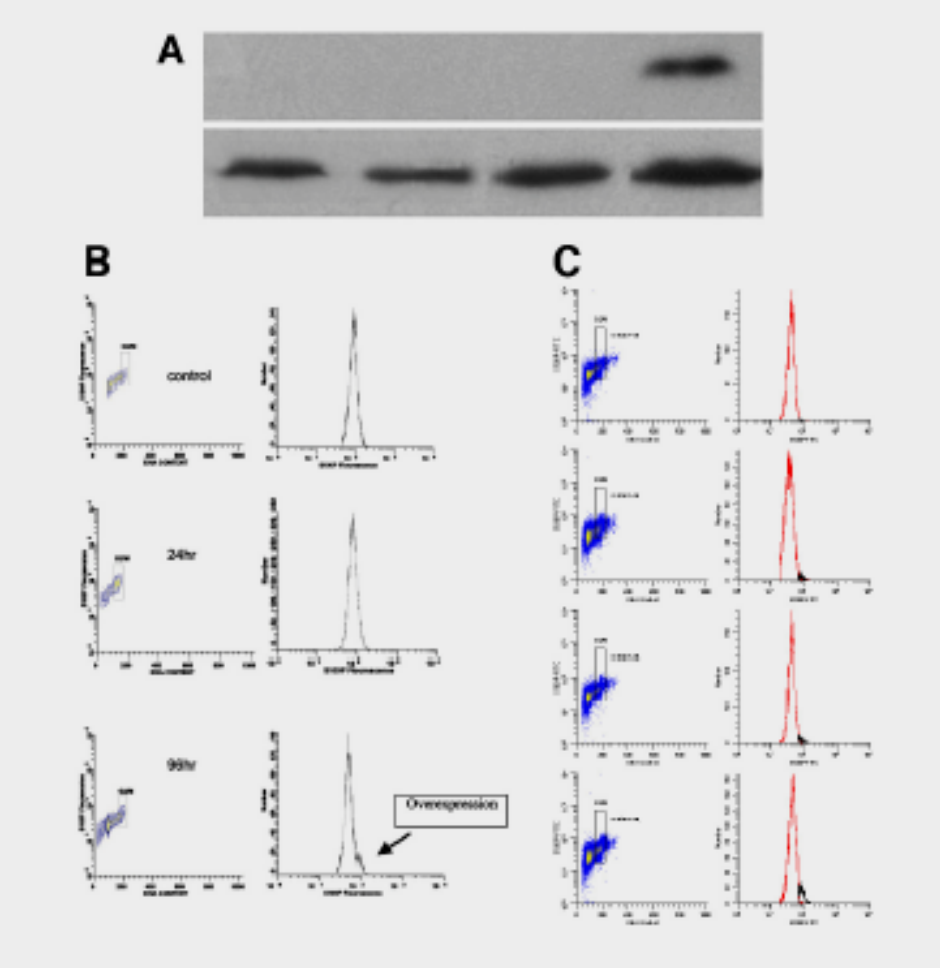


Figure III-3. Upregulation of S100P protein/antigen in HL-60 cells following addition of HN_2. A. Immunoblot detection of S100P (upper panel) in whole cell protein extracts prepared from untreated cells (lane 1) and cells treated for 45 (lane 2), 70 (lane 3) and 90 hours (lane 4). The internal control beta-actin signal for the same samples (lower panel). B. Flow cytometric analysis of S100P and DNA of untreated (control) and cells exposed for 24 and 96 hours to 1.0 μM HN_2. C. Flow cytometric analysis of S100P and DNA in untreated HL-60 cells (upper panel) and cells exposed to 0.8, 1.0 and 1.2 μM HN_2 (lower three panels) for 96 h. In both B. and C. the panels on the right show S100P levels with increases (right shoulder) at 96 hours (lower right panel in B.) and with 0.8, 1.0 and 1.2 μM HN_2 (lower right panels in C).

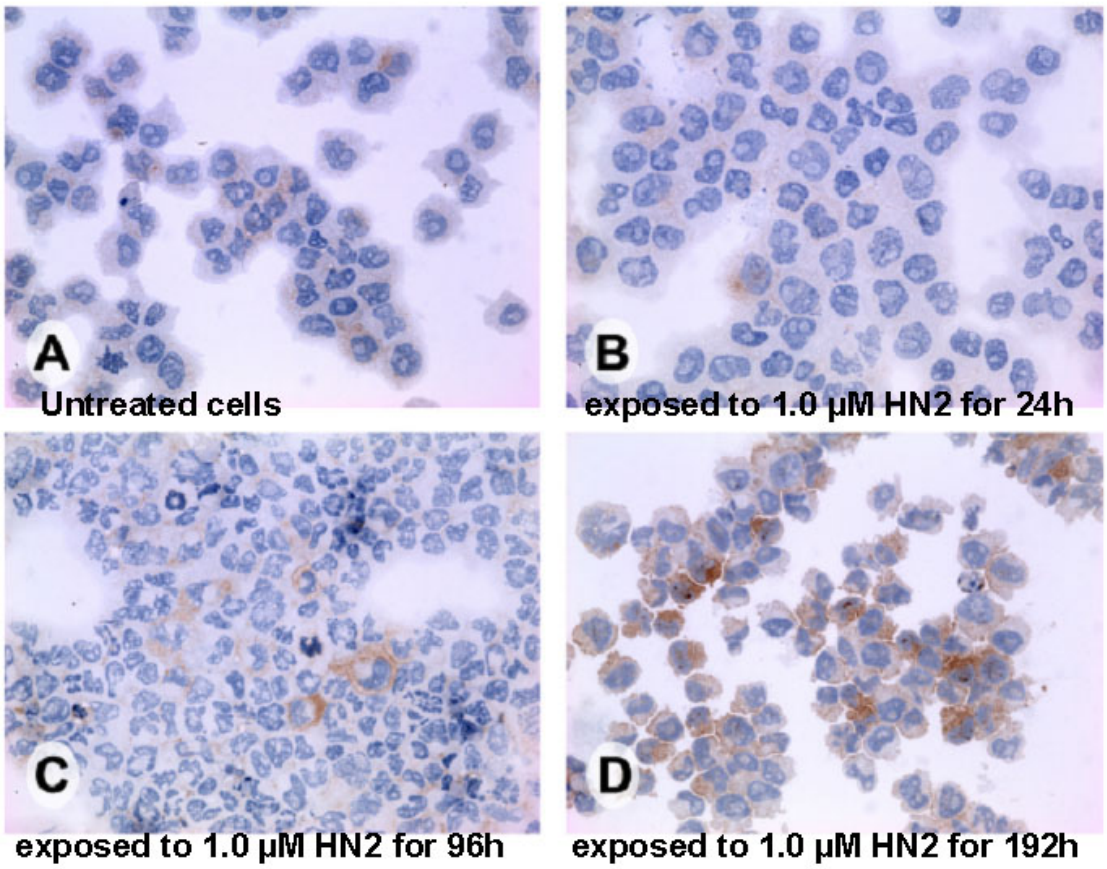


Figure III-4. Immunocytochemical detection of S100P in HL-60 cells exposed to HN_2. Untreated cells (A), and cells exposed to 1.0 µM HN_2 for 24 (B), 96 (C) and 192 (D) h. The immunoperoxidase reactivity shows a cytoplasmic localization. Original magnification: 400X.

IV.

S100P mRNA REGULATION IN RESPONSE TO HN_2 CHALLENGE IN OTHER TUMOR CELL LINES

Introduction

To determine if S100P upregulation with HN_2 treatment is specific for HL-60 cells or is a general response in cell lines, S100P expression in MCF-10F was tested, a breast cell line, with HN_2 treatment. The MCF-10F cell line is a non-tumorigenic epithelial cell line. It retains all the characteristics of normal epithelium *in vitro*, including anchorage dependence, non-invasiveness and non-tumorigenicity in nude mice (Soule, Maloney et al. 1990). The response of S100P in these cells may closely represent the responses in normal epithelial cells.

Two non-hematopoietic tumor cell lines, HBL-100 (breast cancer) cells and Colo 320 HSR (colon cancer) cells were also tested. S100P has been reported to be differentially expressed among normal breast tissue and breast cancer cell lines. A colon cancer cell line was chosen because S100P is expressed in normal colon tissue, though the expression level is low. In addition, there is a report showing S100P overexpression in ulcerative colitis, a condition that predisposes to development of colon cancer (Lawrance, Fiocchi et al. 2001).

Two other hematopoietic cell lines that were also tested, U-937 and K562. The U-937 cell line is a monocytic myeloid cell line, derived from cells obtained from the pleural effusion of a patient with a histiocytic malignancy (Kong, Miwa et al. 1990). As a monocytic cell line, it was chosen to represent myeloid cell lines. K562 cells (chronic myeloid leukemia

with the translocation BCR-ABL) (Boldt, Kopecky et al. 1994) were also used to represent more primitive and multipotent hematopoietic tumor cell lines.

Different cell lines have different sensitivities to HN_2. There is published information on the IC50 for HN_2 in the above cell lines (Sunters, Grimaldi et al. 1998). A wide range of doses covering the IC50 to treat cells was used, to ensure including a dose having toxicity similar to a 1μM dose in HL-60 cells, and to determine HN_2 dose dependency of S100P expression in other cell lines. S100P expression at a series of time points was also followed after HN_2 challenge, if varying dosage failed to show a difference in expression.

Materials and Methods

Cell lines. K562 (human chronic myeloid leukemia), U937 (human acute monocytic leukemia) cells, MCF-10F (nontransformed breast cell line) cells, HBL-100 (breast cancer) cells, and Colo 320 HSR (colon cancer) cells were purchased from American Type Culture Collection (Rockville, MD). They were cultured and maintained as instructed. , They were passed twice weekly, and prepared for experimental procedures when in log-phase growth (cell density 4 x 10^5 cells/ml).

Results

S100P is induced by HN_2 in U937 and Colo 320 HSR cells. The myeloid cell lines U937 (human monocytic leukemia), K562 (chronic myeloid leukemia), MCF-10F (nontransformed breast cell line), nonhematopoietic cell lines HBL-100 (breast cancer), and Colo 320 HSR (colon cancer) were tested. For each cell line, HN_2 concentrations including the published IC50 were used, and total RNA was extracted at the indicated time points after

treatment. The expression of S100P mRNA at baseline was constitutively high in both U937 cells and K562 cells, very close to the placental control level and hematopoietic cells. In K562 cells S100P mRNA did not increase after HN_2 treatment at any of the time points tested (Figure IV-1a, Figure IV-2). In U937 cells, the IC50 of HN_2 was 4.1 μM. S100P mRNA levels increased 2.2 and 3.9-fold at 51 and 74 hours after 3 μM HN_2 challenge in U937 cells (Figure IV-2).

S100P baseline levels in HBL-100 and Colo 320 HSR were relatively low compared to the placental control (Figure IV-1.b). In Colo 320 HSR cells, at HN_2 concentrations of 0.25, 0.5, 0.75, 1, 2, and 3 μM, S100P mRNA levels increased 2, 5, 6, 6, 15, and 16.5-fold, respectively at 24 hours (Figure IV-1 b, Figure IV-2). S100P message was not induced in HBL-100 cells with similarly toxic HN_2 doses at series of time points. HN_2 did not alter S100P mRNA in MCF-10F (data not shown) (less than a 2 fold change).

It is concluded that S100P induction by HN_2 is not a uniform feature of cell lines, but is not limited to HL-60 cells, and that high expression of S100P is characteristic of hematopoietic cell lines.

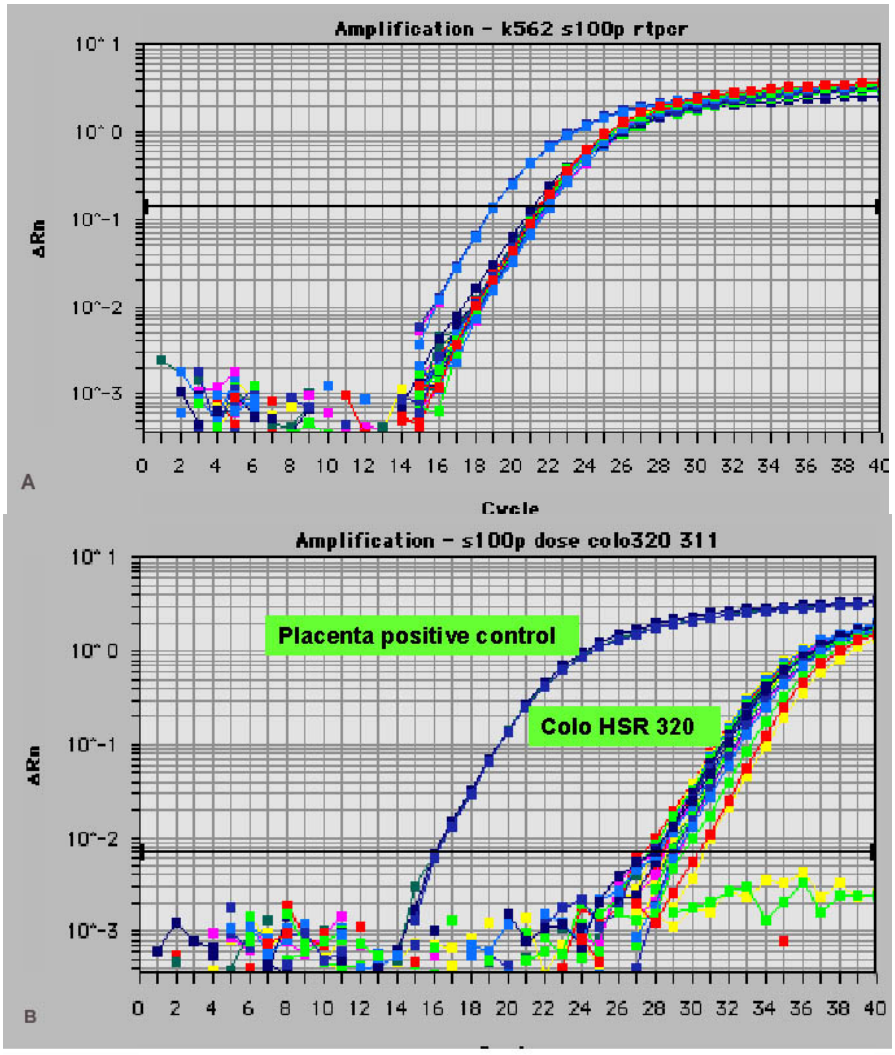


Figure IV-1. S100P mRNA expression in K562 and colo 320 with HN_2 challenge. Total RNA was extracted and S100P mRNA was measured by real time PCR. S100P mRNA is not upregulated by HN_2 in K562 cells (a); S100P is upregulated progressively in colo HSR 320 cells with varying doses of HN_2 (0.25, 0.5, 0.75, 1, 2, 3 μM)(b). Representative amplification plots of real time quantification of S100P in different cell lines. Each includes a plot of the positive placenta control on the left in each figure. ΔRn: normalized reporter signal.

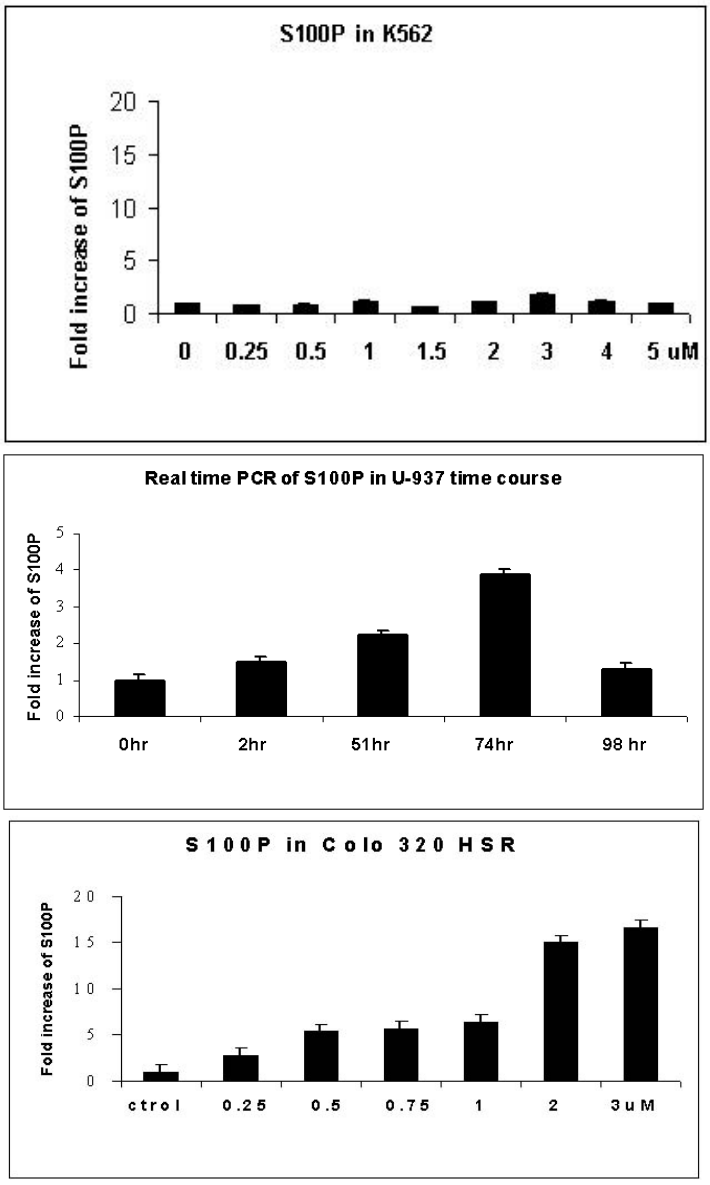


Figure IV-2. S100P is not upregulated in K562 cells with HN_2 treatment at 24 hours; it is up regulated by 3μM HN_2 at 51 and 74 hours; it is upregulated in Colo 320 HSR by HN_2 at various doses. At 24 hours.

Discussion

The goal of this specific aim is to determine if the upregulation of S100P is restricted to HL-60 cells. High baseline S100P expression in the hematopoietic cell lines U937 and K562 was observed. In HL-60 cells, its expression level is intermediate, while the highest level of S100P expression is found in placenta. In nonhematopoietic cells such as Colo320 HSR, MCF-10F and HBL-100, endogenous S100P expression is low. This is consistent with the previously published description that expression of many S100 calcium-binding proteins is tissue and cell-type specific (Donato 2003). S100P is highly expressed in hematopoietic cells, but also expressed in breast, prostate, esophagus and other organs (Jin, Wang et al. 2003). Upregulated S100P expression has been reported in breast, prostate, esophageal and gastric cancers. S100P is more highly expressed in HL-60 cells and hematopoietic cells.

It was found S100P was also upregulated by HN_2 in U937 and Colo 320 HSR cells. As mentioned earlier, S100P has a high basal level in HL-60 cells while Colo 320 HSR cells have a lower basal level of S100P expression. This indicates the upregulation of S100P by HN_2 is not dependent on basal S100P level. The upregulation of S100P by HN_2 is not restricted to HL-60 cells, though the upregulation is not observed in every cell line tested. The underlying mechanism and role of this upregulation remains an area of investigation.

It is not surprising to see S100P upregulation in Colo 320 HSR cells, as S100P mRNA is detected in normal colon tissues (Jin, Wang et al. 2003). Using microarray, genes that are uniquely expressed in Ulcerative Colitis (UC) have been reported; S100P is among the genes that are overexpressed in colon tissue in UC, and UC is a premalignant disease with a high late incidence of colon cancer (Lawrance, Fiocchi et al. 2001). S100P is not upregulated in K562 cells treated with HN_2. It is possible that BCR-ABL protein in K562 may result in activation of different pathways than other cell lines in response to DNA

cross-link damage, not involving S100P. Alternatively, the high level of constitutive expression of S100P in K562 cells may make upregulation of S100P unnecessary in response to DNA cross-link damage. There is also no upregulation of S100P detected in HBL-100 cells.

S100P is not upregulated by HN_2 in MCF-10F cells by HN_2 treatment. MCF-10F is a non-transformed breast cells. It may have an adequate DNA cross-link damage response/repair system without S100P involvement. In addition, epithelial cells may have a different DNA cross-link repair system. However, detailed information of the system is largely unknown. Fanconi patients with genetic defects in DNA cross-link repair, do not have epithelial cell manifestations while they develop bone marrow failure such as MDS and AML (Auerbach 1992; Lensch, Tischkowitz et al. 2003). Thus cell type may contribute to the different response in different cell lines.

In summary, S100P upregulation by HN_2 is not limited to HL-60 cells, S100P upregulation by HN_2 was observed in hematopoietic such as HL-60 and U937 cells as well as non hematopoietic cell lines such as Colo 320 HSR. This suggests S100P may be involved in a relative common pathway of response to DNA cross-linking agents. However S100P upregulation was not observed in all the cell lines we tested. This suggests there may be multiple DNA repair systems are involved in the response to DNA cross-link damage. These different pathways may be tissue specific and they may complement each other. Different pattern activation and inactivation of certain oncogenes and tumor suppressor genes may affect these pathways to different degrees. This may explain the difference of S100P upregulation by HN_2 in these cell lines. For example, S100P upregulation is observed in HL-60 and U937 cells while there is no upregulation in K562 cells. HL-60 cells are p53 deficient while K562 cells are BCR-ABL positive. Their difference in carcinogenesis and

gene expression profile may explain their difference in response to DNA damage. Since S100P upregulation is not restricted t HL-60 cells, the upregualtion carries importance in understanding the mammalian cell response to DNA cross-link damage.

V.

RESPONSE OF OTHER S100 GENE EXPRESSIONS TO HN_2 CHALLENGE

Introduction

S100P is upregulated in nitrogen mustard treated HL-60 cells. The S100 calcium-binding protein family has 21 members to date (Donato 2003). Gene specific sequences for nine members of S100 calcium binding protein family including S100P are also on the microarray membrane. Gene expression of the other eight S100 proteins (S100 A2, A3, A8, A10, A11, A12, A13, B) was not regulated by HN_2 treatment based on microarray analysis. To further determine if S100P is unique among S100 proteins in being upregulated by nitrogen mustard, S100B, S100A8, and S100Z genes were analyzed.

S100B shares more than 50% sequence with S100P (Nowotny, Spiechowicz et al. 2003). S100B has been reported to be expressed in neurons and may play a role in Alzheimer disease (Mrak and Griffinbc 2001) (Figure V-1). Patients with Down's syndrome may develop Alzheimer disease if they survive through adulthood. They can also develop AML. Thus, it would be interesting to test if S100B is also upregulated by HN_2.

S100A8, another member of S100 protein family, was also tested. S100A8 and S100A9 expression in monocytes occurs in early stages of differentiation (Lagasse and Clerc 1988) and in infiltrating monocytes, but not in differentiated tissue macrophages (Zwadlo, Bruggen et al. 1988), suggesting that the expression of S100A8 and S100A9 may be related to differentiation stages of monocytes and macrophages. S100A8 has relative high expression in HL-60 cells (Figure V-2). It has been demonstrated that S100A8 may be involved in inflammation (Passey, Xu et al. 1999).

Finally, S100Z was tested, a novel family member of the S100 calcium binding protein family (Donato 2003). It was found to interact with S100P by the yeast two-hybrid screen (Gribenko, Hopper et al. 2001), suggesting S100P and S100Z may form heterodimers, making S100Z an additional candidate for regulation by HN_2. If S100Z is not upregulated by HN_2, it is still possible it works with S100P as a dimer that is regulated by HN_2 induced damage.

Materials and Methods

Cell lines, Cross-linking agents and monofunctional alkylating agents, RNA extraction, and Real time PCR are as described in previous chapters.

Results

It was found S100B has no message expression in HL-60 cells untreated or treated with 1μM HN_2 (data not shown). The tissue distribution of S100B and S100P are shown respectively (Figure V-1, Figure V-4). These findings indicate HL-60 cells do not express S100B, which is consistent with literature findings, nor is S100B inducible in HL-60 cells by HN_2.

Unlike S100P and S100Z, the S100A8 mRNA level was lower in placenta than in HL-60 cells. The expression of S100A8 wais not regulated by 1 μM HN_2 treatment at different time points (data not shown). S100A8 expression was also tested in HL-60 cells with exposure to varying HN_2 doses of 0.25, 1, 1.5, 2, and 2.5 μM HN2. Total RNA was extracted at 24 hours after HN_2 treatment, and the levels of S100A8 mRNA determined by real time PCR. S100A8 expression was unchanged in each sample relative to the control (Figure V-3).

Finally, S100Z mRNA was analyzed in HL-60 cells that were treated with 1μM HN_2 to determine if S100Z is upregulated by HN_2 treatment. Total RNA was extracted at 1, 2, 6, 24, 48, and 72 hours after treatment, and quantification of S100Z was performed by real-time PCR. Placenta, which expresses higher levels of S100Z relative to HL-60 cells, was used as a positive control, though the baseline S100Z expression is much lower than S100P in placenta. After HN_2 challenge, S100Z mRNA levels increased 2.4-fold over baseline at 6 hours, and increased to 8-fold over baseline at 72 hours (Figure V-5).

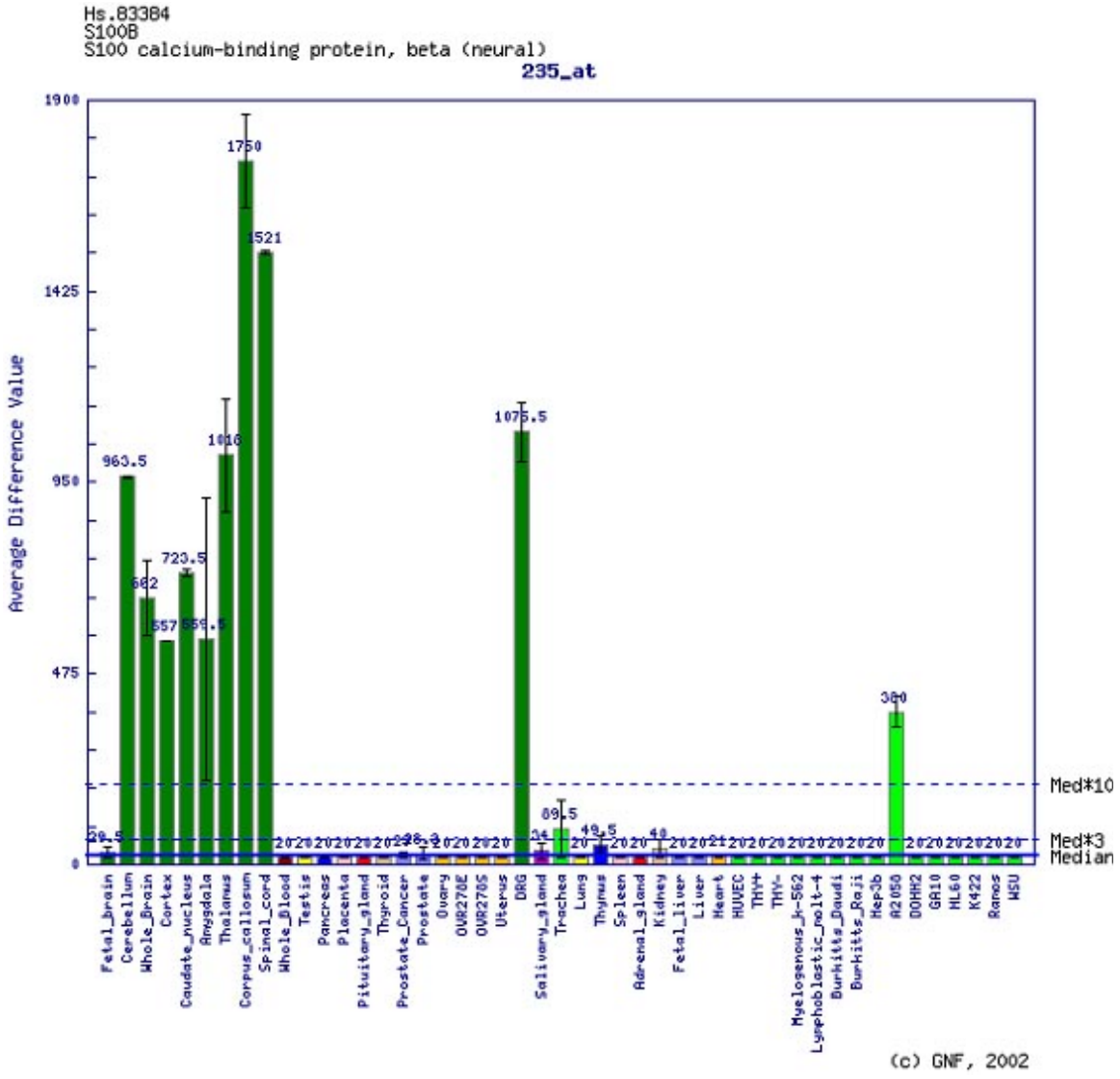


Figure V-1. S100B tissue distribution. (http://expression.gnf.org/cgi-bin/index.cgi#Q)

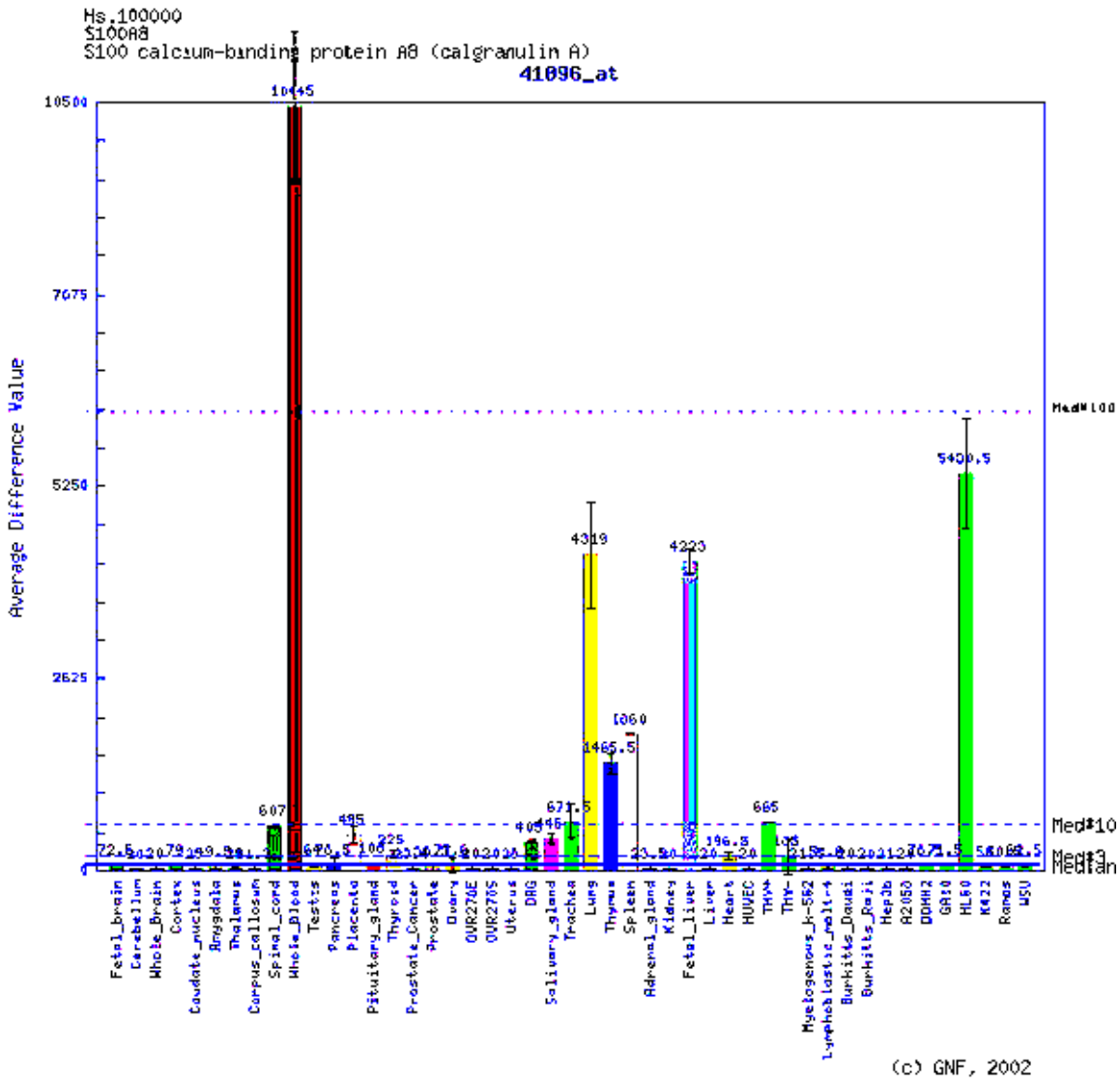


Figure V-2. S100A8 tissue distribution. (http://expression.gnf.org/cgi-bin/index.cgi#Q)

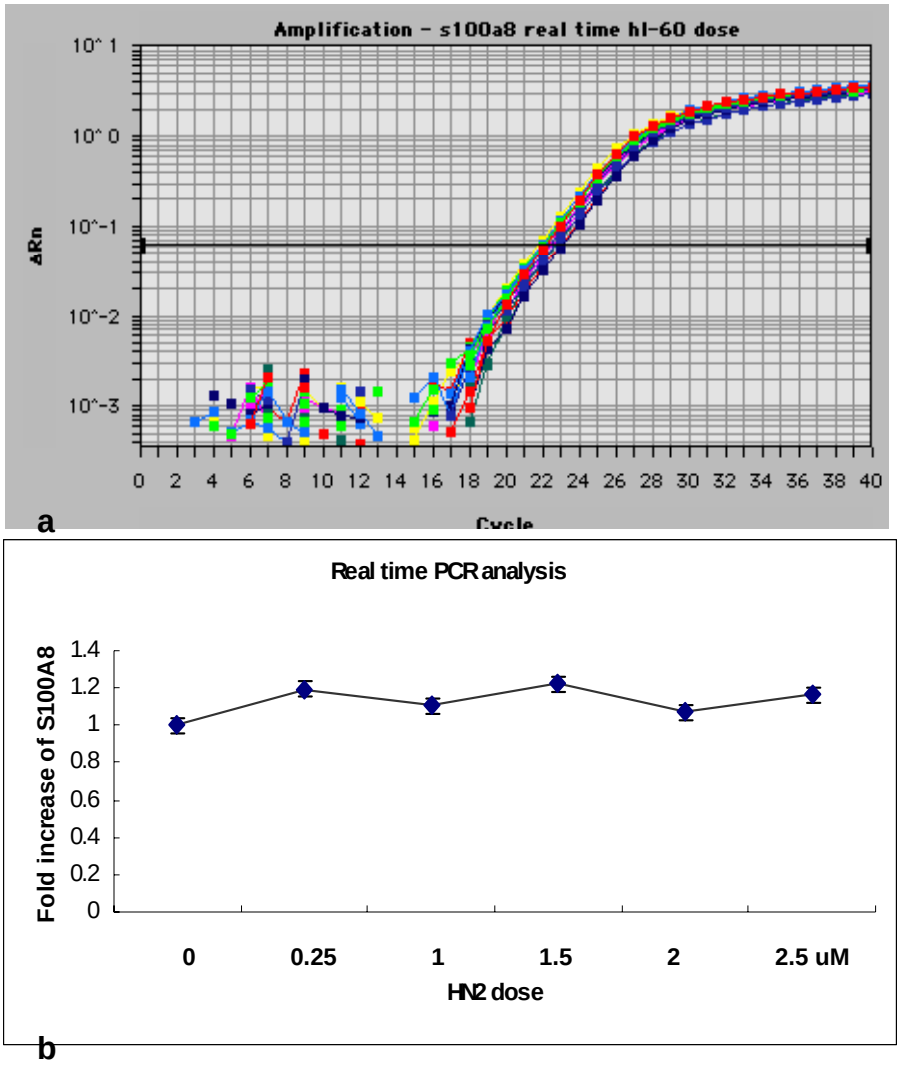


Figure V-3. S100A8 mRNA is not upregulated by HN_2 challenge. Total RNA isolated from HL-60 cells after serial HN_2 dose (0, 0.25, 1, 1.5, 2, 2.5 μM) challenge at 24 hours. S100A8 mRNA level was measured by real time PCR amplification.

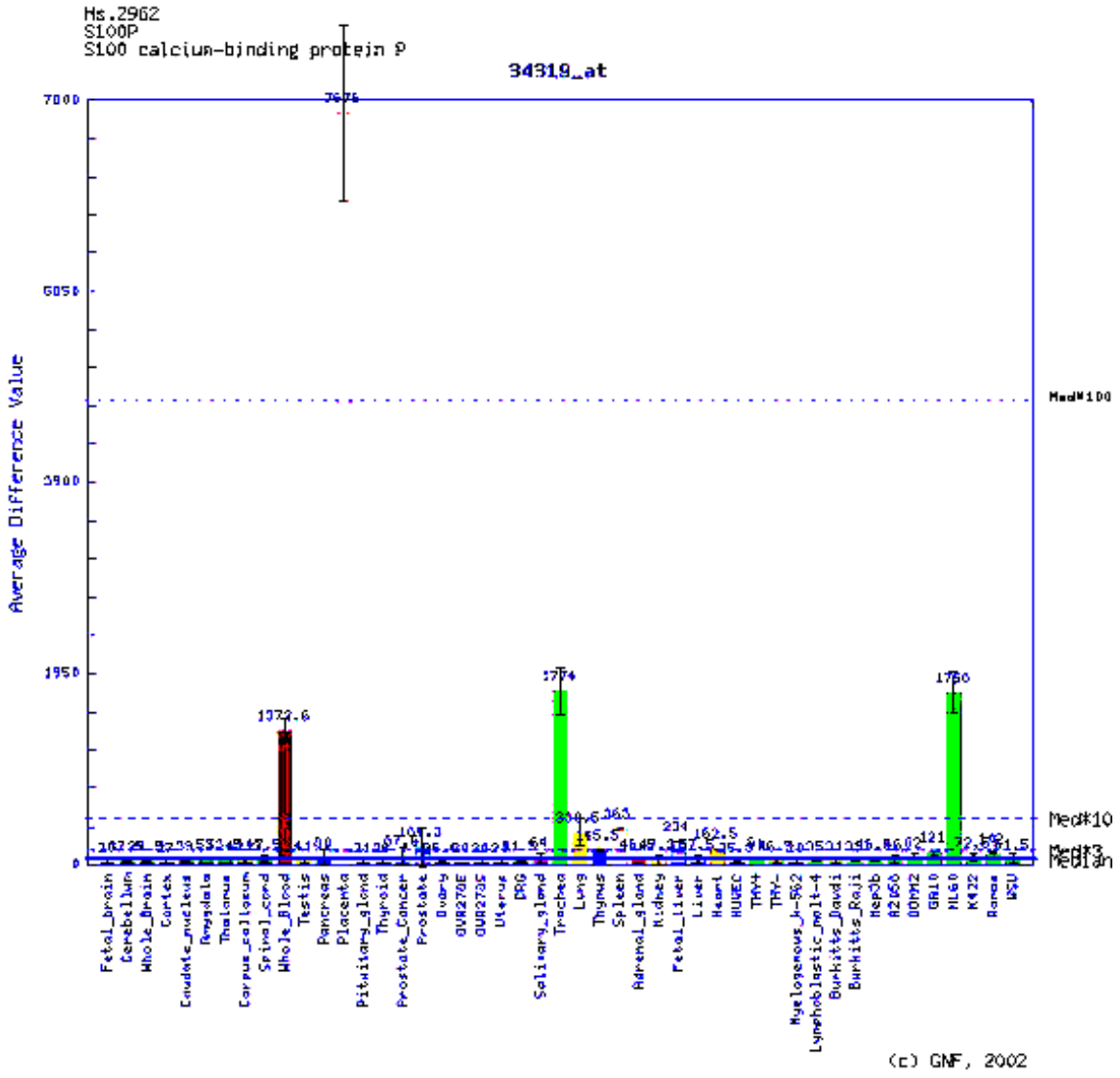
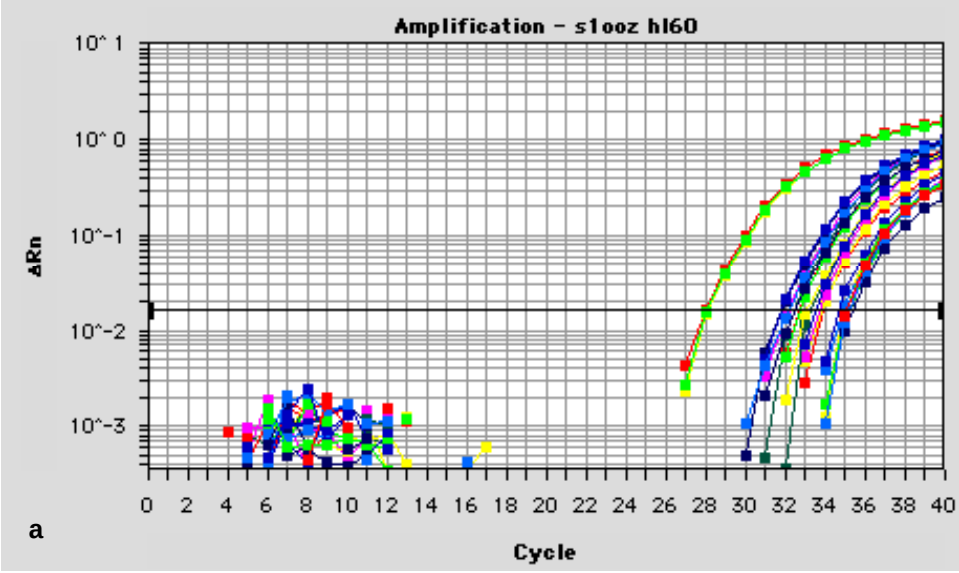


Figure V-4. S100P tissue distribution. (http://expression.gnf.org/cgi-bin/index.cgi#Q)



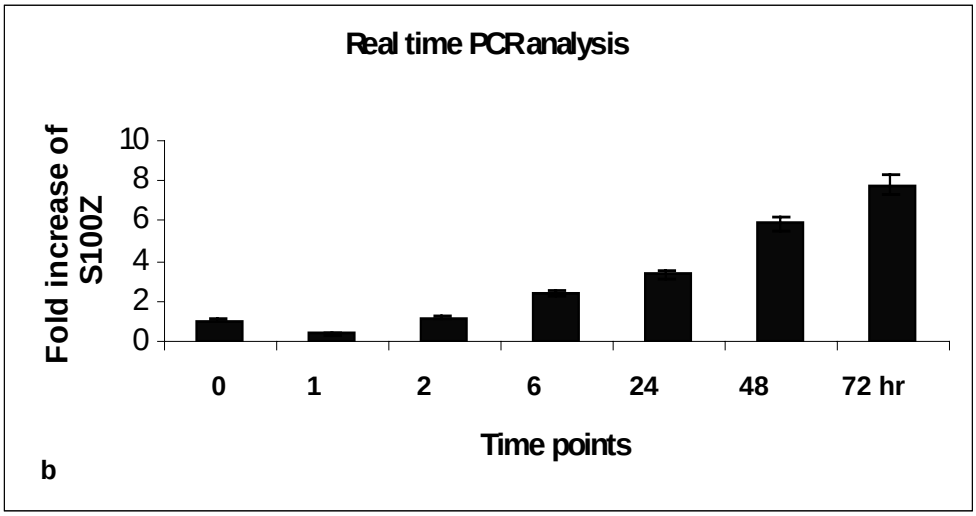


Figure V-5. Upregulation of S100Z mRNA by HN_2 and time dependence. a. At serial time points (0, 1, 2, 6, 24, 48, 72 hours) after 1 μM HN_2 challenge of HL-60 cells total RNA was used for S100Z real time PCR amplification. Each plot represents an amplification curve. b. Data analysis of real time PCR.. The error bars are CVs that were consistently less than 5%.

Discussion

The S100 calcium-binding protein family contains 21 members to date. Most S100 calcium-binding proteins are located on chromosome 1. S100P and S100B are located on chromosome 4 and 21, respectively. Most S100 family proteins function in signal transduction (Donato 2001). Deregulated expression of the genes is found in various cancers. S100P is one of the least studied protein of this family, and its function is unknown. Since we have demonstrated S100P is upregulated after exposure to HN_2, we evaluated other S100 family members' responses to HN_2 challenge.

S100B has 50% sequence identity with human S100P,. S100B forms homodimers similar to S100P. In addition to Ca^{2+}, S100B also binds Zn^{2+} with a binding affinity that is considerably higher than that for Ca^{2+} (Donato 2001; Mrak and Griffinbc 2001; Sorci, Riuzzi et al. 2003). S100B expression is deregulated in the cerebro-spinal fluid in Alzheimer Disease patients, and it has been hypothesized that it may be involved in the pathogenesis in Alzheimer Disease. S100B expression was not detected in HL-60 cells with or without treatment with HN_2. Review of the tissue distribution of S100B shows it is highly expressed in neurons while expression in bone marrow cells is very low (Figure V-1). S100B was not inducible by HN_2 treatment in HL-60 cells.

Expression of S100A8, which has been reported to be highly expressed in leukocytes, involved in chemotaxis and inflammation (Passey, Xu et al. 1999; Zhi, Zhang et al. 2003) was also tested. No upregulation of S100A8 was observed with HN_2 treatment after a time course or with various HN_2 doses. Of the other S100 family members on the microarray templates, none showed different expression in these gene expression profiles. Evidence exists suggesting functions for most S100 proteins, whereas the function of S100P remains unclear. The unique response of S100P to HN_2 among the S100 family indicates its

function may be unrelated to the function of other S100 family members, except S100Z (Gribenko, Hopper et al. 2001).

S100Z forms heterodimers with S100P (Gribenko, Hopper et al. 2001) and is also upregulated by HN_2 treatment. S100Z is a novel S100 family member, found through yeast two-hybrid as an S100P interacting protein. S100Z is a dimeric, predominantly alpha-helical protein that also binds calcium. S100Z mRNA has a similar pattern of expression in tissues to S100P, with highest levels found in placenta, spleen and leukocytes. S100Z gene expression appears to be deregulated in some tumor tissues such as lung cancer (Gribenko, Hopper et al. 2001). Both S100P and S100Z have two calcium binding domains with different binding affinities. The upregulation of S100Z by HN_2 similar to S100P suggests S100P and S100Z may be co-regulated in response to cross-link DNA damage, and potentially participate as hetero-dimers in a cellular response to this damage. How S100P expression changes relate to S100Z expression changes remains to be elucidated.

In summary, the response of S100P and S100Z to DNA cross-linking agents is unique among the tested S100 calcium binding protein family members. The two genes may be coregulated in response to HN_2, and may function as a heterodimer. Their precise function remains unknown.

VI.

S100P mRNA REGULATION IN REPONSE TO OTHER CROSS-LINKING AGENTS IN HL-60 CELLS

Introduction

S100P is upregulated by the bifunctional alkylating agent nitrogen mustard but not by monofunctional alkylating agents. To determine whether this response is general to cross-linking agents, S100P expression in HL-60 cells treated with other cross-linking agents (cisplatin, MMC, and melphalan) was analyzed. DNA cross-linking agents can cause monofunctional alkylation, interstrand cross-links, intrastrand cross-links, and DNA protein cross-links; although the interstrand cross-links appear to cause the major cytotoxic effect. Based on the proportion of different cross-links, DNA cross-linking agents can be divided into predominant interstrand cross-linkers and predominant intrastrand cross-linkers such as cisplatin. The DNA cross-linking agents include mustard family agents such as nitrogen mustard and melphalan, and non mustard family agents including MMC and cisplatin. The structures of melphalan (Figure VI-1), MMC (Figure VI-2), and cisplatin (Figure VI-3) are shown.

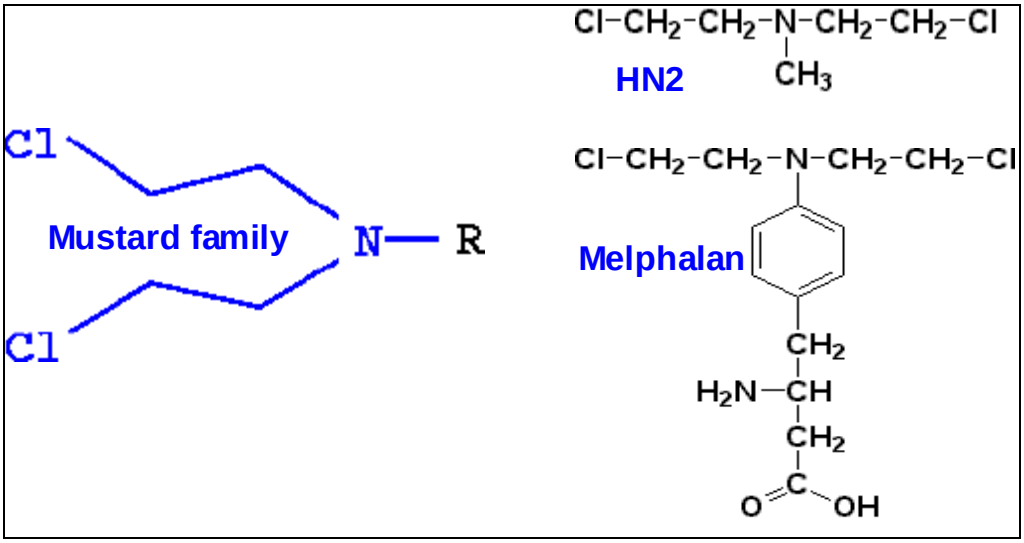


Figure VI-1. Structures of melphalan and mustard family.

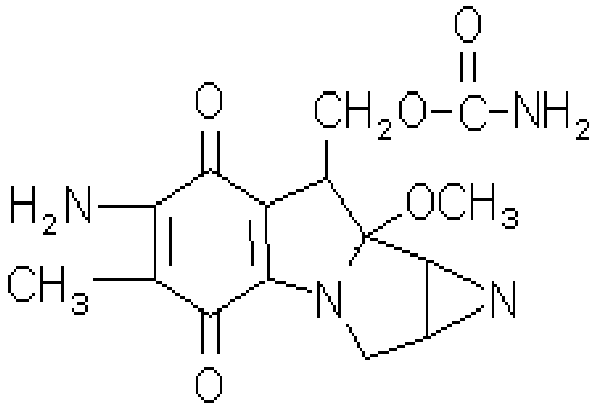


Figure VI-2. Structure of mitomycin C.

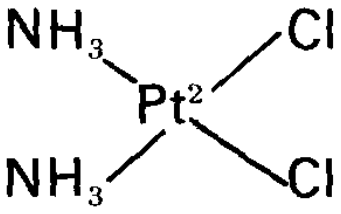


Figure VI-3. Structure of cisplatin.

Materials and Methods

Cross-linking agents. Analytical melphalan and MMC were obtained from MP Biomedicals (Irvine, CA USA). Cisplatin was obtained from Sigma (St. Louis, MO USA).

RNA extraction. Cells in log-phase growth with and without melphalan, MMC and cisplatin challenges, were harvested from 5 ml cultures. Total RNA was prepared using RNeasy® Mini Kits (Qiagen Inc. Valencia, CA) and used for virtual Northern blot and real time quantitative PCR.

Results

To determine whether the induction of S100P mRNA expression is unique to HN_2 or occurs with other agents that cause cross-link DNA damage, S100P induction in HL-60 cells was tested after treatment with the cross-linking agents melphalan, MMC, and cisplatin. Concentrations of melphalan used to treat HL-60 cells were 0, 2, 3, 4, 5, 6, 7, and 8 μM. After 24 hours, S100P mRNA levels increased up to 3.3-fold in cells treated with 3 to 8 μM melphalan (Figure VI-4). HL-60 cells were also treated with MMC (IC50 = 50 nM) at concentrations from 5 nM to 1 μM. After 48 hours, S100P levels by real time PCR increased 3, 11, 13, 13, 15, and 7.5-fold with 5 nM, 25 nM, 50 nM, 100 nM and 1 μM MMC respectively (Figure VI-5). S100P mRNA levels increased 20-fold in HL-60 cells 72 hours after treatment with 1.25 μM cisplatin (Figure VI-6), a dose with a similar toxic effect to 1 μM HN_2 on HL-60 cells by MiCK assay analysis and cell cycle flow cytometry (data not shown).

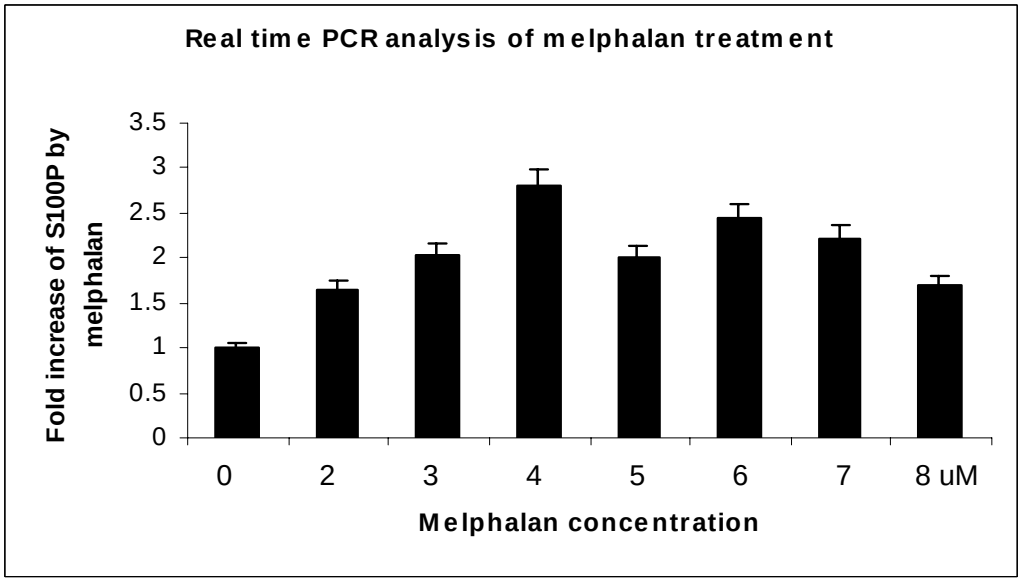


Figure VI-4. Upregulation of S100P mRNA by melphalan. A series of melphalan doses (2, 3, 4, 5, 6, 7, 8 μM) was used to treat HL-60 cells. Total RNA was extracted 24 hours after treatment and used for real time RT-PCR amplification.

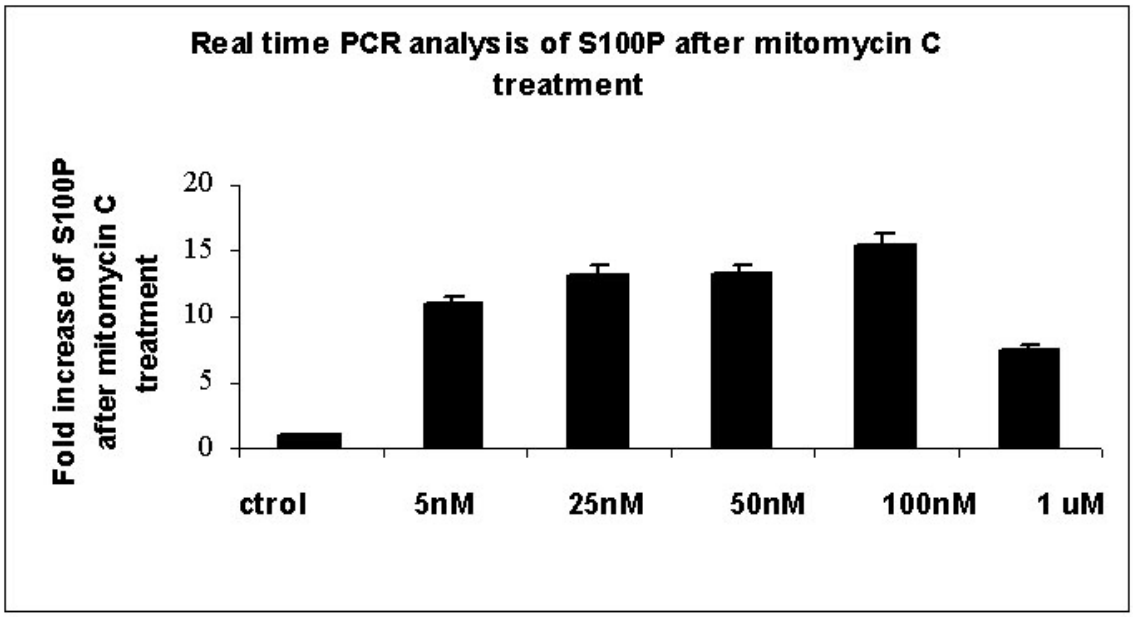


Figure VI-5. Upregulation of S100P mRNA by MMC. A series of MMC doses (control, 5, 25, 50, 100 nM, 1 μM) were used to treat HL-60 cells. Total RNA was extracted 48 hours after treatment and used for real time RT-PCR amplification.

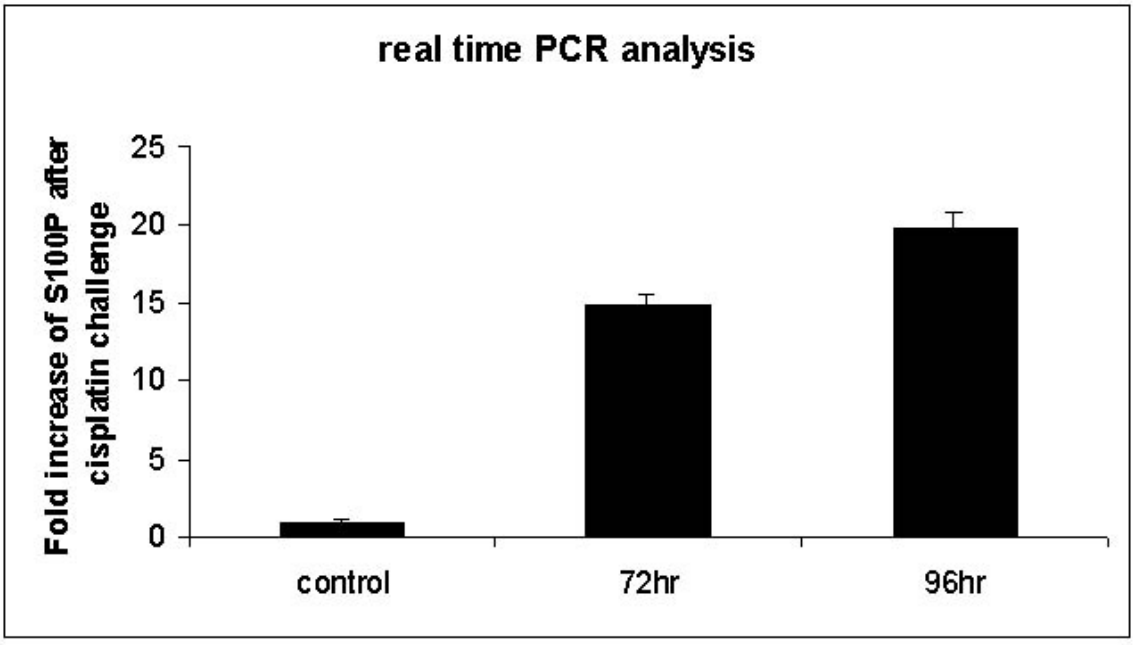


Figure VI-6. Upregulation of S100P mRNA by cisplatin. A series of time points (control, 72 and 96 hours) were followed on HL-60 cells treated with 1.25 μM cisplatin. Total RNA was extracted and used for real time RT-PCR amplification.

Discussion

To determine if the upregulation of S100P by HN_2 in HL-60 cells is unique to HN_2 or a general response to agents that cause cross-link damage, HL-60 cells were challenged with other cross-linking agents and the expression level of S100P was tested. S100P induction was seen in HL-60 cells treated with all cross-linking agents (melphalan, MMC and cisplatinum) tested.

Interstrand cross-links (ICLs) constitute up to 5% of the DNA damage caused by nitrogen mustard and its derivatives. These ICLs cause a major distortion of DNA 3-D structure (Larminat, Zhen et al. 1993; Wiencke and Wiemels 1995; Lawley and Phillips 1996; Dronkert and Kanaar 2001). Nitrogen mustards cause guanine monoadducts (next to the N-7 guanine ICLs in the sequence GpNpC) and DNA–protein cross-links, and they also react with RNA. Melphalan also causes N-3 adenine adducts (Lawley and Phillips 1996). Mitomycin C, a natural antitumor antibiotic, forms adducts at the N-7 and N-2 position of guanine, intrastrand cross-links, and ICLs between the N-2 position of guanines at d(CpG) sequences in the minor groove (Pritsos and Sartorelli 1986; Tomasz 1995). Both nitrogen mustards and MMC mainly form ICLs. In contrast, cisplatin mainly forms intrastrand cross-links (Chu 1994). Cisplatin reacts with guanines to form diadducts in DNA, resulting in 65% d(GpG) intrastrand cross-links, 25% d(ApG) intrastrand cross-links and 5–8% ICLs between the guanines in the sequence d(GpC). The structure of a cisplatin ICL incorporated in an oligonucleotide has been determined and reveals a major distortion of DNA 3-D structure. The platinum is located in the minor groove of the DNA. The helix displays a 47° bending towards the minor groove and a 110° unwinding, while cytosines are extruded from the helix (Larminat, Zhen et al. 1993; Chu 1994; Lawley and Phillips 1996).

All DNA cross-linking agents that were tested upregulate S100P. Mitomycin C and cisplatin strongly induce S100P. The difference of the ability to upregulate S100P may be secondary to the time points that are tested. Some cross-linking agents may upregulate S100P at a relatively early time point while others may to that at later time points. The difference of location of DNA cross-link may also play a role in the difference.

Thus, S100P upregulation is not restricted to nitrogen mustards or dependent on the type of agent causing DNA cross-link damage, suggesting S100P may be involved in a generic response to DNA cross-links.

VII.

CELLULAR BEHAVIOR AFTER HN_2 TREATMENT AND EXPLORATION OF POSSIBLE FUNCTIONS OF S100P

To evaluate the possible function of S100P, the cellular response of S100P induction to DNA cross-link damage may provide clues. When mammalian cells are treated with DNA cross-linking agents, they develop cross-link damage of DNA, arrest the cell cycle and undergo DNA repair (Murray and Meyn 1986; Mu, Bessho et al. 2000; Sancar, Lindsey-Boltz et al. 2004). If the damage is too severe to repair, cells undergo apoptosis. Since the study of cell death is not the goal of this project, we used a dose of cross-linking agents that caused minimal cell death. If cells are able to repair the damage, they may reenter the cell cycle and undergo proliferation (Shima, Munroe et al. 2004; Dendouga, Gao et al. 2005), or as a result of the insult they may undergo differentiation (Gerson, Berger et al. 1992). Thus analyzing the cellular responses to DNA cross-link damage such as cell cycle distribution, drug resistance, cell differentiation, and proliferation could be helpful to understanding a possible role of the induced S100P protein.

HN_2 causes a G2 arrest at 24h and cell growth delay in HL-60 cells

Human myeloid leukemia HL-60 cells were treated with a range of HN_2 concentrations in order to establish a maximum tolerated dose. One micromolar HN_2 did not induce extensive cell death [<10%, determined by detection of cells with sub-G1 DNA content and MiCK assay (data not shown)]. At 24 hours after addition of 1 μM HN_2, an obvious late S/G2 arrest was detected by flow cytometric analysis of DNA content (Figure

II-2). At later time points (48, 72 and 96 hours), the cell cycle DNA distribution returned to that of untreated cells (Figure II-2). The effect of HN_2 on the number of cells produced in the culture appeared as a delay at one day consistent with the transient cell cycle block observed at 24 hours and as an extended phase of reduced rate of cell growth (Figure VII-1). Despite the cell cycle return to a pattern similar to a random cycling population by 96 hours as measured by DNA content, the rate of cell growth did not return to that observed in the untreated cells until two weeks after initiating HN2 treatment. The total number of viable cells in HN2 (1 μM) treated cultures did not drop below the starting level at any time, consistent with minimally induced cell death.

Discussion

S100P mRNA starts to increase 24 hours after HN2 treatment, when HL-60 cells appear to be arrested at S/G2 phase. Interestingly, at this time point S100P protein expression in HL-60 cells remains close to the control. Cell cycle arrest at G2 24 hours after HN2 treatment is a unique response of HL-60 cells to HN2 treatment. This phenomenon was not observed when HL60 cells were treated with similar chemotherapeutic agents that don't cause DNA cross-link damage (DME/DEE). Upregulation of S100P mRNA at twenty-four hours and its relationship to cell cycle arrest is still unclear. This biological role of S100P remains to be investigated. The apparent G2 arrest induced by HN2 begins to resolve by 48 hours, but theS100P protein is not significantly upregulated until 96 hours after HN2 treatment, at which time the cell cycle by DNA content has returned approximately to normal. 4 days after HN2 treatment, cells increase the growth rate compared to the first 4 days after treatment. Around 2 weeks after treatment, cells are proliferating at a rate similar to control cells without any treatment. This recovery could be explained by the dilution of

the original treated cells by passaging in culture. We did not study the kinetics of the return of S100P to baseline levels to determine when induced S100P is back to baseline. However, as described in the next section, ICC reveals that S100P protein levels remain high at 196 hours post-HN2 treatment. It should be noted that a cell cycle arrest is present at 24 hours post treatment but by 96 hours cells have completely recovered from G2 arrest. As mentioned above, S100P protein does not become upregulated until 96 hours, suggesting that S100P does not play a role in the G2 arrest that is initiated at 24 hours. However, these results may indicate S100P promotes cell cycle progression from G2 arrest.

This hypothesis could be tested in several ways, one involving attempts to knock down protein levels and another attempts to overexpress S100P protein. In the first case, siRNA against S100P could be used to knock down the induction that occurs post drug treatment. If S100P rescues the cells from the G2 arrest then the siRNA treated cells will not recover from their arrest. Conversely, if S100P is constitutively overexpressed, the cells may never go into a G2 arrest because S100P is driving the cell cycle away from arrest. The overexpression experiments may require an inducible system if the high stable overexpression of S100P is toxic to cells. Alternatively the gene can be transiently transfected into cells along with a sortable cell surface marker such as CD8, and the transfected population could be sorted and analyzed (van den Heuvel and Harlow 1993). Finally it cannot be ruled out other molecules together with S100P or independent of S100P may play a role in cell cycle progression. For example, induction of p53 may be a cause of the transient cell cycle arrest observed at 24 hours post drug treatment. Ultimately the experiments involving direct manipulation of S100P levels, such as siRNA knockdown or overexpression, will be most informative for determining S100P function. Of course transgenic and knockout mouse models could also be useful for this purpose.

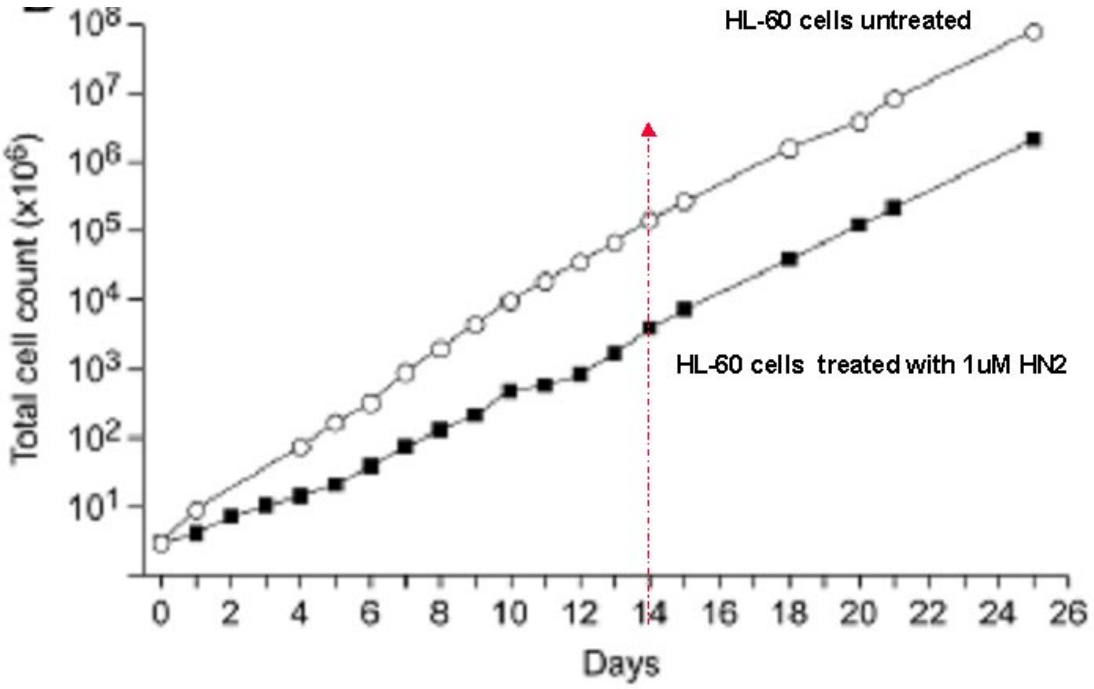


Figure VII-1. Effects of 1.0 μM HN_2 on HL-60 cell growth. Total number of cells produced in untreated (○) and HN_2 treated (■) cultures. Average counts from three flasks were plotted and the CVs averaged 12%.

Rechallenge of HN_2 treated HL-60 cells

HL-60 cells that had been exposed to HN_2 for 2 weeks or 4 weeks were reexposed to HN_2 and cell growth compared to cells that had not been exposed previously to HN_2. Both cultures that were rechallenged with HN_2 showed resistance to the growth suppressing effects of HN_2 over the 14 d period (Figure VII-2). HL-60 cells were then re-treated six months after the initial exposure to HN_2 and showed no resistance to the growth suppressing effects of HN_2 (data not shown).

Discussion

The above findings suggest HL-60 cells with initial exposure gained resistance to HN2 rechallenge to some degree. However this gained resistance does not protect cells completely from DNA cross-link injury since cells with preexposure do not grow as well as the group without rechallenge. These findings are observed in HL-60 cells treated two or four weeks before. Introduction of growth factors from fresh media along with the passaging of the cultures may contribute to the growth curve difference. Interestingly, S100P protein was found to be expressed in HL-60 cells at 196 hours after HN2 treatment through immunocytochemistry.

Six months after initial exposure to DNA cross-linking agents, HL-60 cells do not show resistance to HN2 treatment when compared to the cells never exposed to HN2 previously. Though the molecular pathogenesis of the resistance is unknown, the resistance is not permanent. S100P expression was also tested around 6 months after HN_2 treatment. There is no difference of S100P expression in HL-60 cells challenged 6 months previously and cells without any previous treatment. How long S100P expression lasts and how it is regulated remains unclear.

From the experiments described above, it is not known if S100P is continuously upregulated. There is no direct evidence that suggests or rules out that S100P is involved in drug resistance. As mentioned previously, a more extensive time course of S100P induction would be informative. This would be both a time and labor intensive process. In addition, the constant introduction of new media over a 6-month period may not reflect the pure effect of DNA cross-linking agents on S100P in HL-60 cells, which is the main goal of further study.

In addition, a so-called resistant HL-60 cell line was established after multiple treatment of HL-60 cells with escalating doses of HN_2. This resistance to HN_2 was not a stable phenotype and this cell line is no longer available. S100P mRNA and protein was tested in "resistant" and control cell lines. There was no significant difference between these two groups. A possible role in drug resistance could explain why S100P is upregulated in various cancers (Gros, Ben Neriah et al. 1986). But we did not see an upregulation of S100P, suggesting that S100P is unlikely to play a major role in drug resistance.

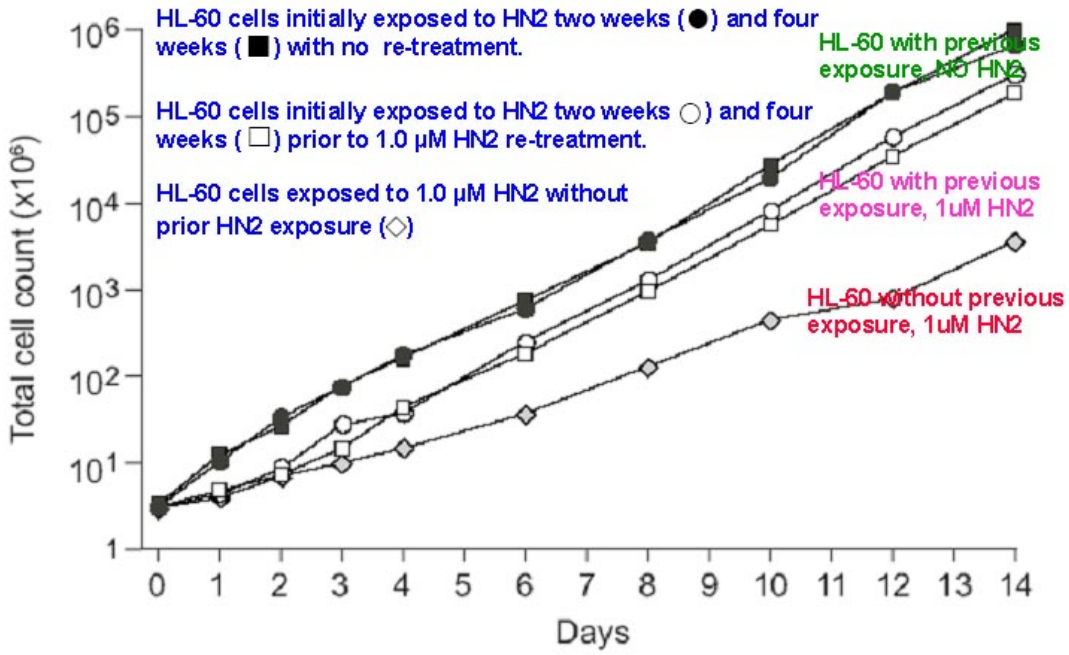


Figure VII-2. Effects of re-exposing HL-60 cells to 1.0 µM HN_2 on cell growth. HL-60 cells initially exposed to HN_2 for two weeks (●) and four weeks (■) prior to no re-treatment. HL-60 cells initially exposed to HN_2 after 2 (○) and 4 weeks (□) prior to 1.0 µM HN_2 re-treatment. HL-60 cells exposed to 1.0 µM HN_2 without prior HN_2 exposure (◊). The results are representative of three experiments.

Karyotype analysis

A karyotype analysis was completed on 20 metaphases from HL-60 cells exposed to HN_2 for 14 d. Three karyotypes were detected (Table 2) indicating that the culture was not clonal after exposure to 1 μM HN_2.

The analyses of HL-60 cells 14 d after HN_2 revealed three karyotypes, which is inconsistent with a clonal selection during the delayed recovery phase following HN_2 exposure.

Discussion

From previous experiments, HL-60 cells gain resistance to a DNA cross-linking agent challenge to some degree two weeks or four weeks after HN_2 treatment.

Drug resistance can be gained through many different mechanisms. One of them is selection of a single resistant clone after multiple treatments. This single clone with mutations that help cells to be resistant to drug treatment are selected because of their resistance.

HL-60 cells treated with HN2 gained resistance two weeks after treatment. Karyotype analysis at that time fails to detect a single clone that contributes to the resistance. In fact, three karyotypes were detected. This indicates that single clone selection is not the mechanism involved in the drug "resistance " in HL-60 cells treated with HN2 since two karyoypes were present before treatment. The mechanism of appearance of the third karyotypes is not clear. Given that the HL-60 cells were challenged with a mutagenic DNA cross-linking agent, DNA damage may have contributed to the development of the new karyotype.

Table 2. A karyotype analysis was completed on 20 metaphases from HL-60 cells exposed to HN_2 for 14 d and untreated HL-60 cells.

Karyotype analyses

	# of karyotypes/20 metaphases	
Karyotpe [a]	HN_2 14 da.	Untreated
1	11	11
2	3	9
3	6	0

[a] Karyotypes:

1 = 47,X,X,del(9)(p12),add(11)(q23),add(16)(q24)x2,+18,add(19)(q13.4),+mar1

2 = 49-50,idem,+der(1;7)(q10;p10),del(10)(p13),mar1,+mar2,+mar3,+mar4

3 = 49,idem,del(1)(q23),+der(1;7)(q10;p10),del(10)(p13),-mar1,-mar2,+mar3,+mar4

S100P and cell differentiation

Introduction

S100P is expressed during tumor progression in breast cancer (Guerreiro Da Silva, Hu et al. 2000; Mackay, Jones et al. 2003) and prostate cancer (Averboukh, Liang et al. 1996; Gribenko, Lopez et al. 1998; Amler, Agus et al. 2000; Mousses, Bubendorf et al. 2002). S100P is expressed in the early stage of esophageal epithelial cell differentiation (Sato and Hitomi 2002; Zhi, Zhang et al. 2003). S100P expression is also tissue specific (Jin, Wang et al. 2003), selectively expressed in bone marrow, esophagus, prostate, and colon. The potential role of S100P in differentiation and tumorigenesis may relate to the induction of S100P after exposure to agents that cause cross-link DNA damage.

Since S100P is highly expressed in HL-60 cells 96 hours after HN_2 treatment, it would be interesting to test the differentiation status of HL-60 cells at that time point. CD11b is a commonly used surface marker for monocytes (Hurwitz, Raimondi et al. 1992; Scott, Head et al. 1994). It was tested if this population started differentiation towards monocytes by detecting the cell surface marker CD11b. HL-60 cells at time 0 and 96 hours without treatment and cells at 96 hours after HN2 treatment were tested for expression of CD11b.

Materials and Methods

Antibodies and agents. Primary Anti-S100P monoclonal mouse antibody was purchased from the BD biosciences (Transduction Laboratories, Lexington, KY). Anti CD-11b PE antibody was kindly provided by Esoterix (Brentwood, TN).

Quantitation of CD11b positive cells protein by flow cytometry. Cellular CD11b was measured in HL-60 cells from 0 to 96 hours after 1 μM HN_2 treatment. Cells were washed twice in PBS +2% FCS and fixed in 70% EtOH in the dark at room temperature for 1 hour. Cells were then washed twice in cold 1X PBS. A saturating amount (as determined by titration on HL-60 cells) of S100P and CD11b antibody were added and allowed to incubate in the dark at room temp for 1 hour. A matching concentration IgG1-Isotype tube was also prepared in the same fashion. Cells were washed twice in PBS + 2% FCS. A FITC-labeled IgG1-goat anti-mouse antibody at 1 μg/μl was added to S100P, CD11b and isotype tubes and incubated for 1 hour. Cellular DNA was stained with 7-AAD. An FC500 cytometer (Beckman-Coulter Fullerton, CA) with a 488 nm and 633 nm excitation source was used for data acquisition. S100P and CD 11b data were analyzed using WinList 5.0, ModFit LT 5.0 (Verity Software House, Topsham, Maine) and Excel 5.0 (Microsoft, Seattle WA). S100P expression was determined by comparison of each time point to the background protein expression as determined by the isotype control in drug treated and control samples. This method was also used to measure the frequency and intensity of S100P expression in each cell cycle state. Cell cycle data was determined by modeling in ModFit LT and analyzed using Lysys software (Becton Dickinson).

Results

CD11b is a cell surface marker for monocytes. Cell surface staining of CD11b was performed in nontreated HL-60 cells at zero and 96 hours, and HL-60 cells at 96 hours after challenge with HN_2. These HL-60 cells were also stained with S100P and 7AAD. CD11b was not detected in the majority of cells in these three groups, suggesting an absence of monocyte differentiation in non-treated and treated HL-60 cells. Thus there is no evidence that upregulation of S100P stimulates monocytic differentiate of HL-60 cells. A more definitive evaluation of a role for S100P in differentiation of HL-60 cells would require specific ablation of S100P mRNA with siRNA as described in section I discussion.

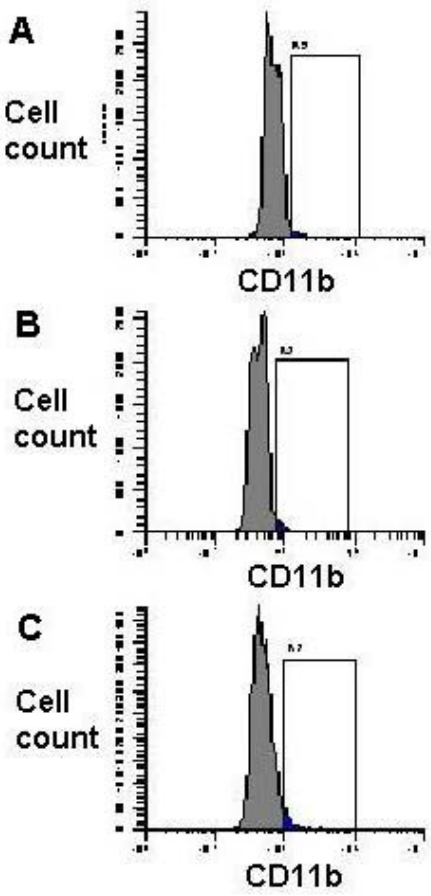


Figure VII-3. CD11b expression did not change significantly in HL-60 cells without or with HN_2 treatment. HL-60 cells with or without treatment were followed for four days. They were stained with CD 11B, S100P, IgG1 isotype. Row A: HL-60 cells without treatment at time 0. Row B: HL-60 cells without treatment at time 96 hours. Row C: HL-60 cells with HN_2 treatment at time 96 hours.

Discussion

Our data showed an absence of CD11b staining in majority of HL-60 cells indicative of monocytic differentiation in HL-60 cells 96 hours after HN_2 treatment. At 96 hours after HN_2 treatment, S100P protein overexpression becomes evident by Western blot analysis, although no cell differentiation is detected. S100P expression was detected in specific layers of esophageal epithelial cells (Sato and Hitomi 2002) and may be involved in differentiation of these cells. The data suggest that S100P induction by HN_2 does not cause monocytic differentiation of HL-60 cells.

To test further a possible relationship between cell differentiation and S100P overexpression, one could determine S100P expression after induction of macrophage differentiation of HL-60 cells with phorbol ester. Elimination of S100P message through siRNA could also be employed to determine if S100P is required for differentiation of HL-60 cells. However, because of its short length and homology to other S100P gene, attempts to define an effective siRNA have not been successful to date. The role of overexpressed S100P in HL60 differentiation could be studied in the same system previously described for studying cell cycle effects. In fact, arrest of the cell cycle is often associated with cellular differentiation, so we hypothesize that S100P does not stimulate differentiation. This is also consistent with the upregulation of S100P found in cancers, where undifferentiated, uncontrolled growth was observed.

S100P and cell cycle distribution

Introduction

DNA cross-linking agents such as HN_2 affect, cell cycle arrest (Murray and Meyn 1986; O'Connor, Wassermann et al. 1991; O'Connor, Ferris et al. 1992), DNA repair (Murray and Meyn 1986; De Silva, McHugh et al. 2000), apoptosis (O'Connor, Wassermann et al. 1991; Marathi, Howell et al. 1996), differentiation and proliferation (Hansson, Edgren et al. 1988; Scharer 2005). There is a G2 cell cycle arrest at 24 hours after HN_2 challenge. At later time points (48, 72 and 96 hours), the cell cycle distribution resembles a random, non-synchronized population.

S100P mRNA upregulation starts at 24 hours and maximizes around 96 hours. S100P proteins are upregulated at 96 hours. These findings suggest S100P is not involved in the G2 cell cycle arrest appearing at 24 hours. In fact on the contrary it may promote cell cycle progression from G2 to M. There is no significant sub G1 population appearance in 96 hours course from cell cycle analysis by flow cytometry, which indicates no significant cell death at four days after treatment. This suggests S100P is unlikely to be directly involved in cell death. It may help cell survival by promoting cell cycle progression. To study S100P distribution in the cell cycle, S100P expression during the cell cycle in PHA driven normal lymphocytes was examined. The expression of S100P in HL-60 cells was also checked without any drug challenge.

To analyze the cell cycle distribution, 7-AAD was used to measure DNA content, and anti-MPM2 monoclonal antibody to label the mitotic population. Like Propidium Iodide (PI), 7-AAD was used to quantitate the DNA content after having cells

permeabilized. HL-60 cells and PBMCS were triply stained with S100P, 7-AAD and the mitosis-specific mAb, MPM2, to identify if the mitotic population overexpressed S100P.

Material and Methods

Cells and agents. PHA driven peripheral blood mononuclear cells (PBMCS) were collected from four donors. S100P was studied throughout the normal cell cycle by driving PBMCS with 10 μg PHA/ml media and assaying at 0, 24, 48, 72 and 96 hours in culture. This process was repeated twice with the same 4 donors assaying for S100P and DNA content at each time point.

Cell cycle analysis. 2×10^6 cells stimulated with PHA were collected and fixed in 5 ml ice-cold 70% ethanol, and stored at -20°C overnight in the dark. The ethanol was removed by washing with PBS, and the cells were incubated in 500 μl 7-AAD for 30 minutes at room temperature in the dark. Flow cytometric analysis of DNA content using 7-aminoactinomycin D (7-AAD) was completed using a FACScan analyzer (Becton Dickinson, Mountain View, CA). Cell cycle analysis was conducted using ModFit cell cycle software and WinList histogram software (Verity Software House, Topsham, ME). Apoptotic cell death was quantified as the proportion of cells in the population with a subdiploid (<2N) DNA content. Mitotic population was labeled by the mitotic marker MPM2.

MPM2 and S100P immunostaining for flow cytometry has been described earlier (Chapter VI)

Results

To find the normal distribution pattern of S100P in different stages of the cell cycle, peripheral blood mononuclear cells (PBMCS) were analyzed. Treatment of PBMCS with

PHA was used to induce cell cycling. It was found that S100P expression increased through G1 and S; maximizes at G2, dropped by a half log in mitosis, and then halves as cells complete division, repeating the same cycle (Figure VII-4).

S100P expression was also tested during the cell cycle in HL-60 cells without any drug challenge (Figure VII-5). Similar to the findings in the PBMCS, the mitotic population, which was labeled by MPM2, expressed low levels of S100P. The G2 population has the maximal amount of S100P expression while early G1 appears to have the lowest expression. In addition S100P and MPM2 were analyzed in HL-60 cells treated with HN_2. Cells with 4N DNA content over expressing S100P did not stain for MPM2. Thus the subpopulation with S100P overexpression was not in mitosis but rather consisted of cells in G2. Cells with 4N DNA content staining with MPM2 had low levels of S100P.

Cell cycle distribution in the HN_2 treated HL-60 cells is similar to untreated HL-60 cells, except as observed previously, a G2 cell cycle arrest occurs 24 hours after HN_2 challenge (Figure VII-7). Cell cycle distribution returns to control levels at 96 hours after treatment, coinciding with S100P protein induction.

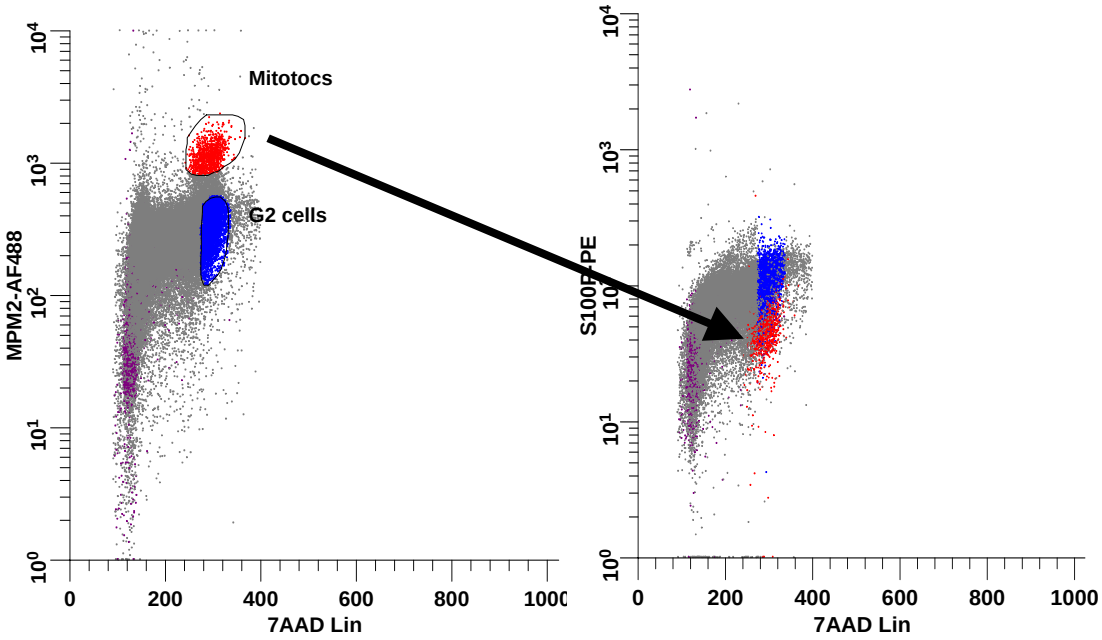


Figure VII-4. Normal S100P distribution pattern at different cell cycle stage of PBMCS driven by PHA

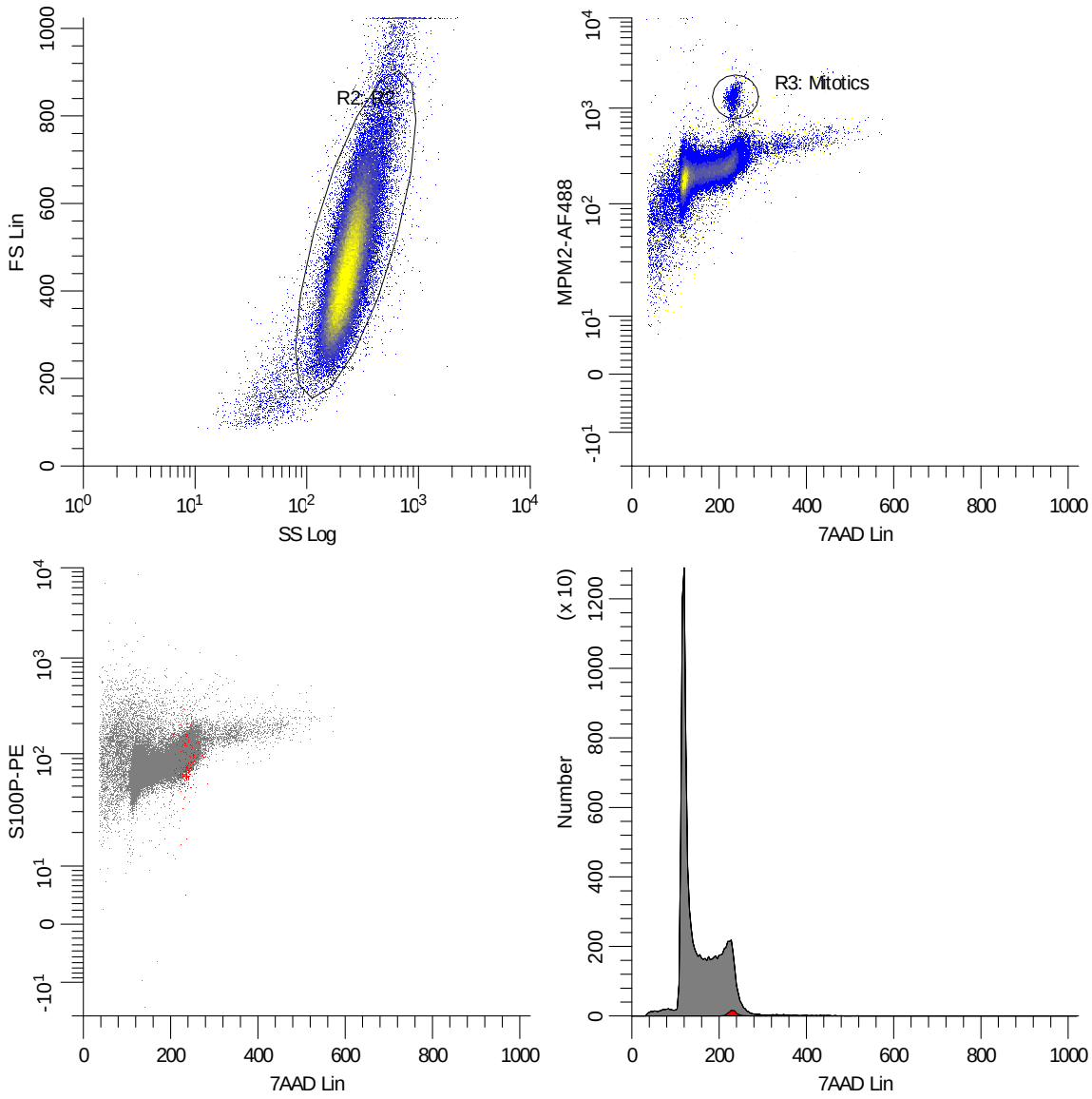


Figure VII-5. S100P distribution in HL-60 cells without drug challenge. Mitotic population (red in bottom panels) does not overexpress S100P.

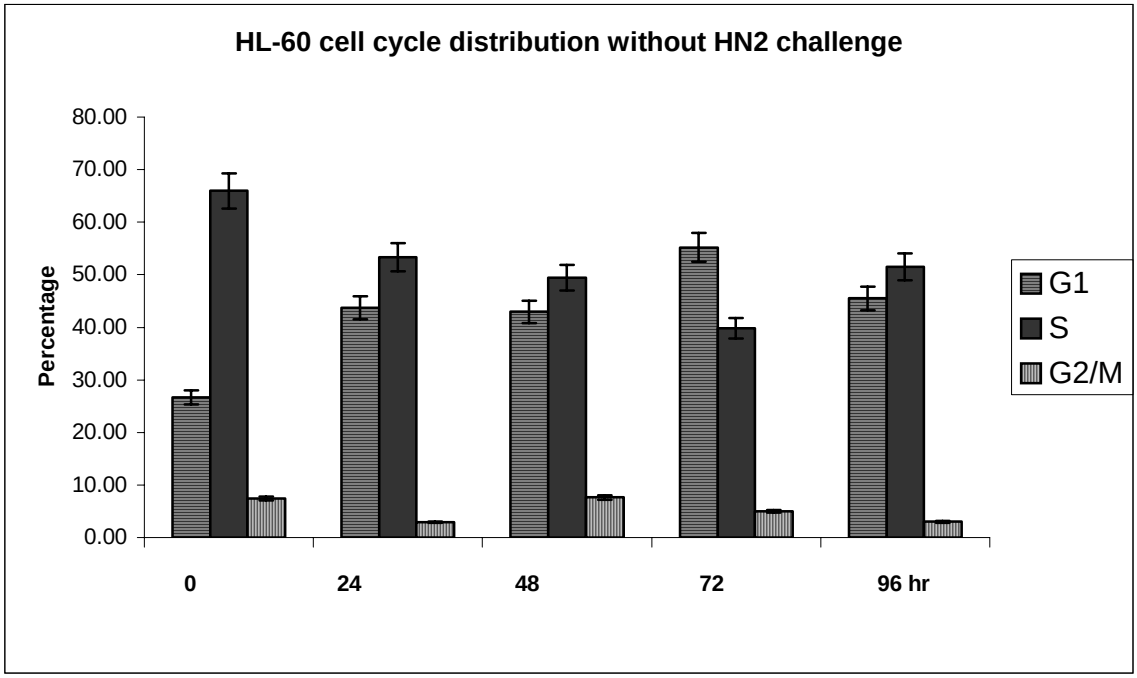


Figure VII-6. The cell cycle distribution in HL-60 cells up to 96 hours without HN_2 challenge.

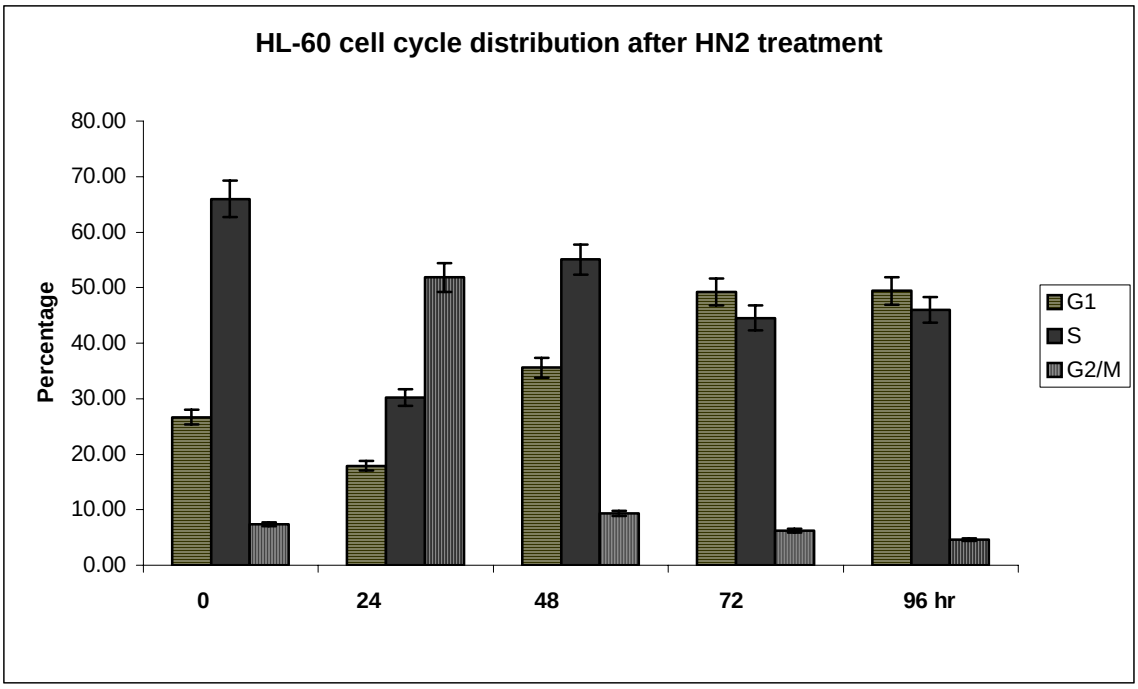


Figure VII-7. The cell cycle distribution in HL-60 cells up to 96 hours after HN_2 challenge

Discussion

S100P distribution in normal PBMCS is high in G2 and low in mitosis. "Caretaker" proteins double their protein content from G1 to G2, and decrease by 50% as they progress through mitosis to G1. In PBMCS, S100P expression in the G2 cell population is approximately ten fold greater than the lowest G1 level, and is much more than the two-fold ratio of caretaker proteins. The abrupt decrease of S100P in mitosis suggests a role for S100P in cell cycle regulation, possibly as a checkpoint gene or a trigger for apoptosis; while down regulation of S100P may permit cell cycle progression through mitosis.

This hypothesis is supported by the finding of S100P upregulation in HL-60 cells treated with HN_2. As shown in Figure VII-7, at 24 hours after HN2 treatment, cells are not cycling and there is a significant G2 arrest. This period of time is when S100P is upregulated only by RNA analysis, followed by protein upregulation by Western blot and immunohistochemical analysis at 96 hours. It is possible that degradation of S100P is required for cell cycle entry into mitosis in this setting. Other interpretations are possible, and as it is clearly important to do both protein overexpression and protein knockdown to define the role of S100P in mitosis and cell cycle progression. As is stated in the Conclusion section, S100P is only found in vertebrates, so cell cycle progression in a basic sense does not require S100P. The caveat is that some fine aspect of cell cycle control in vertebrates could have evolved to require S100P function.

S100P protein is upregulated around 96 hours when cells appear to have recover ed from the G2 cycle arrest that initiated at 24 hours. By ICC at 196 hours it is apparent that S100P remains elevated, and this suggests that the elevation persists for an extended time beyond 96 hours. There is no significant sub-G1 population to suggest cell death is

occurring in the S100P elevated population. Therefore, S100P is not likely to contribute to cell death. Its overexpression in the G2 population in HL-60 cells suggest it may play a role in cell survival, and because we don't know if these cells are cycling it may actually be promoting a cell cycle arrest to allow for recovery from DNA damage. However, it may also be promoting cell cycle recovery from arrest. Again, the only way to clearly define the role of S100P is to overexpress the protein, similar to what occurs after drug treatment, and note whether the cells undergo cell cycle arrest or stimulation of proliferation.

S100P and cell proliferation

Introduction

A recent publication indicates S100P can stimulate proliferation of NIH3T3 cells. These cells do not normally express S100P protein. NIH3T3 cells stably expressing S100P were produced using standard transfection techniques (Arumugam, Simeone et al. 2003). S100P expression increased the proliferation rate of NIH3T3 cells to 251 ± 42% that of control ($n = 3$, $p < 0.05$) within 72 h. Extracellular S100P also stimulated NIH3T3 cell growth and survival. In these experiments histidine-tagged S100P was expressed and purified from *E. Coli*. Non-induced bacterial proteins were used as a control. The addition of purified S100P (0.01–1000 nM) to NIH3T3 cells stimulated cell proliferation in a concentration-dependent manner. Effects were noted with 0.01 nM, and maximal effects (188 ± 23% of control, $p < 0.05$, $n = 3$) were observed with 100 nM S100P. The effects of S100P on cell proliferation were also time-dependent, with a significant increase in cell proliferation noted within 48 hours after the addition of 100 nM S100P to the culture medium (Arumugam, Simeone et al. 2004).

The possible proliferation stimulation effect of S100P requires further study. S100P is not naturally expressed in NIH3T3 cells. In addition, this study reported that S100P protein is secreted into the media in S100P transfected cells. In this thesis research project, S100P protein in the media of HL-60 cells was not detected, which produce endogenous S100P.

Although we used the same ELISA assay to detect S100P secretion as described (Arumugam, Simeone et al. 2004), lack of sensitivity for ELISA or the occurrence of false negatives cannot be completely ruled out. It was decided additionally to test if the media from the HL-60 cell culture after HN_2 treatment has any effect on cell growth. If they do, it will be tested if addition of purified S100P proteins has similar effect on cell growth.

The media from HL-60 cells at different time points after treatment with HN_2 was added to untreated HL-60 cells to determine if a secreted growth stimulatory factor was present. Cell proliferation curves were compared to the cells treated with either serum free (negative control) or serum containing RPMI (positive control).

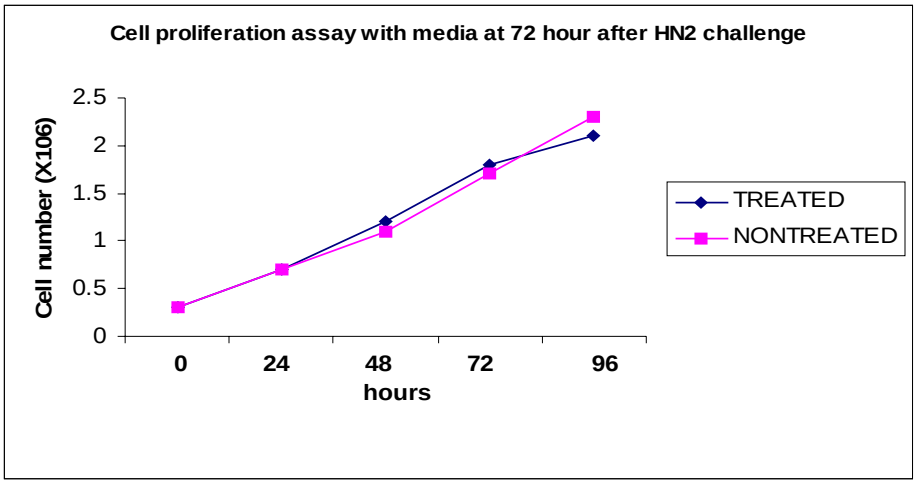
Materials and Methods

Media extraction from cells. HL-60 cells were treated with 1 μM HN_2. Culture was spun down at 24, 48, 72, 96 hours after HN_2 treatment and media was retained. HL-60 cells growing at log phase were prepared for the observation of the effect of the media.

Cell proliferation assay. Cell growth was analyzed by cell counting. For studies on the effects of possible secreted S100P on other cells, media from HL-60 cells treated with HN_2 at different time points was added to HL-60 in RPMI. Control cells were treated with either serum-free RPMI (negative control) or serum-containing RPMI (positive control). Media from non-treated HL-60 cells was also used as control.

Results

Media from HL-60 cells treated with 1 μM HN_2 and from untreated HL-60 cells was collected at 24, 48, 72, and 96 hours, and used to treat HL-60 cells at log phase growth. Serum free RPMI –1640 and serum containing RPMI-1640 were also used as negative controls. Cell counts were recorded daily for 5 days. *G*rowth curves were drawn and compared. As expected, cells treated with serum-containing RPMI proliferated at the highest rate. There was no significant difference between the cell group treated with media from HN_2 treated and nontreated HL-60 cells. Growth curves for HL-60 cells treated with media from HN_2 treated and non-treated cultures at 72 and 96 hours are presented in (Figure VII-8). No obvious difference was observed. Other data are not shown.



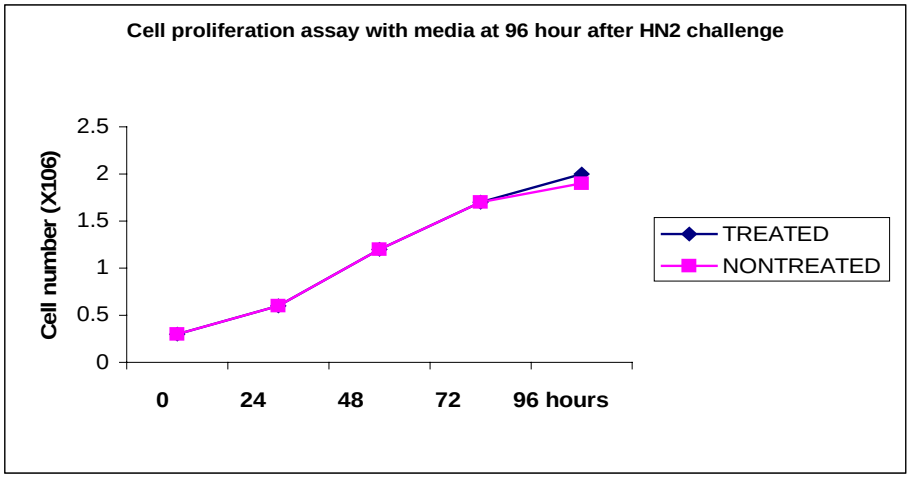


Figure VII-8. Cell proliferation assay using media taken from HL-60 cells at 72 and 96 hours after treatment with 1 μM HN_2 and media from untreated HL-60 cells

Discussion

While the exact function of S100P is not clear, a recent publication reported that S100P was secreted into culture and stimulated proliferation of NIH3T3 cells (Arumugam, Simeone et al. 2003).

As mentioned earlier, no S100P was detected in HL-60 cell culture media by ELISA testing. To eliminate the possibility that the sensitivity of ELISA was not sufficient to detect S100P, the media from HL-60 cells at different time points after treatment with HN_2 was added to untreated HL-60 cells. The added media did not stimulate proliferation of HL-60 cells. Although exogenous S100P reportedly stimulates NIH3T3 cell proliferation, the physiological relevance of this observation is questioned since S100P is not expressed in NIH3T3 cells, and is not secreted from HL-60 cells. In addition, it is unclear whether HL-60 cells have surface receptors for S100P proteins. Lack of receptors, lack of secretion or both may explain the results of the experiments on S100P's role in cell proliferation. In HL-60 cells, RAGE is expressed; Thus lack of RAGE is not the explanation of the test results (van den Heuvel and Harlow 1993).

Another way to test S100P's effect in cell proliferation would be to use a purified recombinant S100P protein. Adding purified S100P protein to HL-60 cells in RPMI, would allow a direct evaluation of the effect of extracellular S100P on cell growth.

VIII.

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Our findings:

To better understand the transcriptional response to cross-link DNA damage, genes were sought that were selectively upregulated in HL-60 cells treated with HN2, but not with two monofunctional alkylating agents (DME, DEE). Monofunctional agents can cause damage similar to HN2, but cannot cause cross-link DNA damage.

Our analysis indicated that the calcium-binding protein S100P mRNA was selectively upregulated by HN2 in a time- and dose-dependent manner. Western blot analysis confirmed the S100P protein level is also elevated in response to HN2 treatment, at a later time than the mRNA level. ICC revealed S100P is localized in the cytoplasm of HL-60 cells. Flow cytometry and ICC also confirmed S100P's upregulation by HN2 at 96 hours, consistent with Western blot data. Flow cytometry also suggests a fraction of G2 population cells overexpresses S100P approximately 96 hours after HN2 challenge, corresponding to a time when cells appear to have completely overcome the G2 arrest that occurred at 24 hours. S100P secretion was not detected in HL-60 cells with or without HN2 challenge.

Upregulation of S100P mRNA by HN2 is also observed in the nonhematopoietic cell line, Colo 320 HSR and in another hematopoietic cell line, U937. All other DNA cross-linking agents tested also upregulated the mRNA level of S100P in HL-60 cells. Therefore,

the up-regulation of S100P by DNA cross-linking agents is neither cell line- nor drug-specific.

Besides S100P, S100Z is also upregulated by HN2. It has been reported that S100P and S100Z can interact with each other through yeast two-hybrid screening. Therefore it is possible that they may function together in response to HN2 challenge. S100B is not expressed or induced in HL-60 cells. S100A8 is highly expressed in HL-60 cells and is not regulated by HN2.

Expression of S100P appears to be higher in some hematopoietic cell lines. It was observed that the baseline expression of S100P mRNA is intermediate in HL-60 cells, and is constitutively high in both U937 cells and K562 cells, very close to the placental control that has the highest expression level of S100P. S100P expression is tissue-specific in normal tissue; it is expressed in leukocytes, trachea, prostate, and bone marrow cells (Guerreiro Da Silva, Hu et al. 2000). Along with other S100 proteins, S100P is exclusively expressed in vertebrates (Kretsinger 1976)), suggesting a role for these proteins in functions found only in higher organisms with endoskeletons. Thus basic cell cycle control may not be a function of S100P or other S100 proteins, because cell division occurs in their absence. Interestingly, organisms did not create a need for the S100 protein family until vertebrates had evolved. Selective high expression of S100P in bone marrow, the calcium binding property of S100P, and evolution of S100P in vertebrates suggest S100P expression may have evolved in parallel with the migration of hematopoiesis to its intramedullary location. The previous findings plus the upregulation of S100P by HN2 and other cross-linking agents in marrow cell lines suggest parallels to the Fanconi (FA) protein system, a system that is also exclusively expressed in vertebrates, and which is involved in the cellular response to cross-link DNA damage and serves an unknown but critical protective role in marrow and bone.

S100P and cell survival:

Although our results do not define a functional role for S100P induction, we do have clues as to how the protein doesn't function, which is important knowledge. Other DNA damage inducible genes such as p53 and Gadd45 (Scharer 2005) will arrest the cell cycle so that DNA damage can be repaired. However, the induction of S100P does not result in cell cycle arrest. Thus S100P is not likely involved in the growth arrest observed when cells are subjected to DNA damage.

S100P may play a role in cell survival. The critical experiment would be to prevent the upregulation of S100P by siRNA in response to sublethal doses of HN_2 and determine the extent of cell death that occurs with siRNA treatment. If S100P is important for survival there will be an increase in cell death upon siRNA knockdown of message and protein under drug treatment. Another experiment would involve enforced expression of S100P; if there is a pro-survival role we would expect to see reduced death in cells overexpressing S100P. Analysis of cell cycle and S100P expression suggests that S100P may be involved in cell survival after HN_2 challenge and regulation of cell passage through S/G2 to M. This provides further directions for research.

The specific increased level of S100P in a subset of G2 cells is an indication that following exposure to HN_2, these cells exhibit a unique response associated with the prolonged recovery phase. A group of cell cycle genes (cdc25c, cyclin A, cyclin B2, cdc2, and survivin), which function to regulate the transition of cells from G2 phase to mitosis, exhibits a similar pattern of G2 specific expression. The G2 specific expression of survivin was recently proposed to counteract a default induction of apoptosis in the G2 phase. Survivin and S100P share similarities related to expression. Both are elevated in many cancers and elevated expression is associated with enhanced survival in cells under stress.

Survivin has been implicated in mechanisms that inhibit apoptosis as well as in chromosome separation and cytokinesis. S100P does not share structural features with survivin nor does it share the same localization within the cell. Normal constitutive survivin is expressed specifically in G2 cells. The constitutive S100P expression was maximal in G2 cells, being at a minimal level in mitosis and in some cells with G1 DNA content (presumably early G1 cells). The level progressively increased as cells replicated DNA. Unlike survivin, mitotic cells expressed a low level of S100P similar to that found in G1 cells. The elevated level of S100P protein (96 hours after HN_2), above the constitutive level, that was detected in cells with G2 DNA content was not associated with growth arrest, as there was no obvious accumulation of cells in a phase of the cell cycle. These cell cycle genes (cdc25c, cyclin A, cyclin B2, cdc2, and survivin) share a number of regulatory elements in their promoter regions including a repressor element termed "cell cycle genes homology region" (CHR). A scan of the sequence upstream of the S100P coding sequence revealed two CHR elements. The possibility that the cell cycle specific expression of S100P is co-regulated with these other genes requires further investigation. The over-expression of S100P detected in a number of cancers and in cell lines capable of enhanced survival under stress supports a role in cellular defense mechanisms. The results are consistent with this, and also indicate that at least in HL-60 cells, S100P function in both untreated cells and particularly in those recovering from the effects of bifunctional alkylating drugs is linked to cells in the G2 phase.

S100P, cell proliferation and anti-apoptosis:

The S100 protein family is the largest subgroup within the superfamily of proteins carrying the Ca2+-binding EF-hand motif. S100 proteins have a plethora of tissue-specific intra- and extracellular functions. Many S100 proteins have extracellular function through

Receptor for Activated Glycation End Products (RAGE) (Donato 2001). It has been shown *in vivo* that S100P can stimulate cell proliferation and cell survival through RAGE (Arumugam, Simeone et al. 2004). The proliferation and survival effects of S100P expression were duplicated in a time- and concentration-dependent manner by the extracellular addition of purified S100P to wild-type NIH3T3 cells and correlated with the activation of extracellular-regulated kinases (Erks) and NF-KB. It was found that S100P co-immunoprecipitated with RAGE. Furthermore, the effects of S100P on cell signaling, proliferation, and survival were blocked by agents that interfere with RAGE including administration of an amphoterin-derived peptide known to antagonize RAGE activation, anti-RAGE antibodies, and by expression of a dominant negative RAGE (Arumugam, Simeone et al. 2004). This suggests the possibility that S100P can act in an autocrine manner in some cells via RAGE to stimulate cell proliferation and survival.

We found no secretion of S100P into the media of HL-60 cells with HN2 treatment, so it is unlikely that in HL-60 cells S100P acts in autocrine manner. However, intracellular functions of stimulating cell proliferation or promoting cell survival are still possible. It will be important to determine the level and activation state of proteins such as Akt, Erk and NF-Kb, which are involved in cell proliferation and survival, when HL-60 cells overexpress S100P. Analyzing these enzymes may provide information that S100P is using these pathways for intracellular signal transduction.

S100P and cancer cell migration and invasion:

Another function of S100P may play a role in migration or invasion of cancer cells. When some cell types are exposed to stressful conditions such as DNA damaging agents or hypoxia the cellular response can include stimulation of cellular movement to exit the

stressful environment and find a more favorable environment. For example the Met tyrosine kinase receptor under conditions of hypoxia will stimulate osteosarcoma cells to migrate (Pennacchietti, Michieli et al. 2003). Expression of the related S100 protein, S100A4, is a poor prognostic factor for breast and colon cancer (Taylor, Herrington et al. 2002). This is consistent with the known role of S100A4 contributing to cell invasion properties (Belot, Pochet et al. 2002; Filipek, Jastrzebska et al. 2002; Mackay, Jones et al. 2003). In support of S100P playing a role in invasion, S100P expression was found to be associated with later stage pancreatic cancer (Rosty, Ueki et al. 2002; Logsdon, Simeone et al. 2003; Sato, Maitra et al. 2003). An *in vitro* method to test S100P in invasion could be to determine if S100P overexpressing epithelial or fibroblast cells can migrate through a Transwell chamber. As well the question could be asked if treatment with HN_2 stimulates the S100P overexpressing cells to migrate or invade. Finally, S100P overexpressing and non-expressing cells can be tested for their ability to form metastases in nude mice. However, it is not clear how these findings affect hematopoietic cells.

DNA damage and S100P regulation

Our results may have implications for the upregulation of S100P that is found in cancer (pancreatic, colon, gastric, breast and prostate cancer). Although S100P is upregulated by bifunctional agents, but not by monofunctional alkylating agents, it is possible that other types of DNA damage, which we have yet to investigate, could upregulate S100P. The global DNA loss/gain that is the hallmark of an aneuploid cancer cell may in fact be a signal that is upregulating S100P (Boige, Laurent-Puig et al. 1997; Marton, Thein et al. 2001).

By contrast it has been shown that DNA mutation is not prevalent in cancer, specifically in colon cancer (Wang, Rago et al. 2002). The results of this study show that DNA mutations are not common in the colorectal cancer genome, occurring on average once every 1Mb or so (Wang, Rago et al. 2002). This mutations frequency is similar to the frequency in normal cells, suggesting that somatic mutations are not occurring at an elevated frequency. It is suggested that these mutations are not a DNA damage signal causing the upregulation of S100P. Therefore, it is possible that the elevated deletion and amplification of chromosomal DNA is a generates a DNA alteration signal that might upregulate S100P.

This could potentially be tested in mouse transgenic models of cancer, where the current mouse models do not typically show the aneuploidy found in human cancer. Alternatively, given our observations we could examine whether patients who had received prior DNA-damaging chemotherapy, upregulate S100P. If patients had received chemotherapy prior to having an analysis of their S100P status, it might complicate whether S100P was upregulated by the cancer cell as part of its normal physiology, or whether the upregulation was due to the response of the cancer cell to chemotherapy. In the study of pancreatic cancer, it was the case that patients had not received prior chemotherapy, thus suggesting that in this cancer the upregulation is not a therapy-induced event.

S100 mouse models:

Transgenic or knock out mouse models of S100P may help to determine S100P's function *in vivo*. There are transgenic or knock out models with other S100 genes such as S100B. Neither S100B inactivation nor overexpression results in abnormalities in mice development. S100B knockout mice show enhanced plasticity in glial cells with enhanced fear memory, suggesting S100B may be involved in information processing. Lack of S100B

resulted in decreased calcium handling capacity in astrocytes (Marenholz I 2004). Increased S100A1 expression resulted in increased myocardial performance under baseline conditions. In an S100A1 knock out model, reduced contraction and relaxation rates of myocardial muscles were observed (Marenholz I 2004). In mouse models, knock out of S100A9 did not show any abnormal phenotypes. Lack of S100A8 led to resorption of the embryo by the mother, indicating a nonredundant lethal function of S100A8 (Marenholz I 2004). S100A4 is a tumor associated S100 protein. Overexpression of S100A4 enhanced the metastatic ability of mammary tumors. Since S100A4 stimulated neovasculization, that may partly explain why S100A4 has tumor promoting function. However, it was found that S100A4 null mice developed more spontaneous tumors than wild type controls, possibly because of destabilization of p53 since S100A4 is found to interact with p53 (Marenholz I 2004). Therefore the cellular context and environment may play a key role in determining the result of S100A4 function, as both overexpression and underexpression can be shown to stimulate aspects of cancer growth.

There are no established knockout or transgenic mice model of S100P. Knocking out or transgenic expression of S100P in mice and analyzing the phenotypes of the mice might be helpful to understand the function of S100P. There are a few possibilities. S100P knock out mice may be lethal. If that is the case, it suggests S100P is a nonredundant protein and is important in development. It may then be attempted to do a conditional knockout in adult mice. S100P knockout mice may not show any obvious phenotype physiologically. Then it may be important to determine the response of S100P knock-out or overexpressing mice to DNA cross-linking agents and the development of any kind of malignancy including hematopoietic malignancies. If those mice do show a phenotype, it can provide direction to study the function of S100P.

S100P, MDS and MDR-AML:

S100P is a novel gene involved in the response to cross-link DNA damage. This observation may be valuable because of the apparent involvement of cross-link DNA damage in the pathogenesis of MDS and MDR-AML. We found that S100P is upregulated by DNA cross-linking agents such as HN_2 in HL-60 cells. Thus it is possible S100P may play a role in the pathogenesis of these groups of diseases. A group from Germany have reported different levels of S100P message between CD34 positive cells from bone marrow in low grade MDS and high grade MDS patients (Hofmann WK 2002). CD34 positive cells were isolated in three groups of patients, including healthy controls, low grade MDS and high grade MDS. Patients with refractory anemia are considered low grade MDS and patients with RAEB are considered high grade MDS. S100P mRNA is expressed at lower level in low grade MDS, compared to healthy controls and patients with high grade MDS.

The protein expression pattern of S100P in myeloid neoplasia, specifically MDS, TDN-AML, and MDR-AML may be tested through ICC or flow cytometry and the RNA expression of S100P in these patients can be tested by in situ hybridization. Further information of S100P expression in the bone marrow of these patients may provide insight to a possible role of S100P in the pathogenesis of MDS and MDR-AML.

Upregulation of S100P expression, a specific response to bifunctional alkylating drugs, may provide new insight into cellular defense mechanisms, cell cycle regulation in response to these agents, and the pathogenesis of both diseases caused by these agents (MDS, AML) and syndromes associated with abnormal responses to these agents (FA, ataxia telangiectasia).

Future Directions

The definitive biological function of S100P is not known. However, many publications suggest possible functions. Researchers have found unique expression patterns of S100P in different cancers, such as breast cancer, prostate cancer, esophageal cancer and pancreatic cancers (Averboukh, Liang et al. 1996; Amler, Agus et al. 2000; Mousses, Bubendorf et al. 2002; Sato and Hitomi 2002; Mackay, Jones et al. 2003; Zhi, Zhang et al. 2003). Others have tried to clarify S100P's function by manipulating its expression in S100P null NIH3T3 cells. S100P protein was found to be secreted in this artificial system. Addition of purified S100P to media containing NIH3T3 cells stimulates cell proliferation (Arumugam, Simeone et al. 2004). Another interesting finding is that S100P is upregulated in doxorubicin resistant colon cancer cell lines (Bertram, Palfner et al. 1998). S100P transfected NIH 3T3 cells were found to tolerate chemotherapeutic agents better than wild type NIH 3T3 cells (Arumugam, Simeone et al. 2004).

Understanding the biological function of S100P will require extensive work. Here I propose a few strategies to understand the function of S100P:

The first is to block S100P expression in cells or cell lines by siRNA. S100P protein expression can be knocked out using RNA silencing technology, by transfecting an RNA expression vector with an appropriate siRNA insert. RNA silencing is a fast and efficient knock out compared with the traditional animal knock out model. However, S100P is a member of a family of 21 proteins. Since the genes for these proteins share close to 50% homology, there are potential technical difficulties in specifically knocking out S100P without affecting other S100 protein genes. After successfully knocking out S100P, the difference in cellular phenotype between the wild type and S100P knock out cells can be

compared, which will provide clues about the function of S100P. Drug resistance to HN_2 and the gene expression patterns of these two cell lines will also be compared, to enable identification of proteins or protein families that are direct or indirect candidate targets of S100P.

The second method is to overexpress S100P in cells or cell lines that lack S100P expression. The difference in cellular phenotype between the wild type and cells with S100P overexpression can be compared, which will provide more clues about the function of S100P. DifferenceS of resistance to HN_2 and the gene expression patterns of these two cell lines will also be compared to enable identification of proteins or protein families that are direct or indirect candidate targets of S100P.

The third method for exploring the function of S100P is to identify S100P interacting proteins. Using immuoprecipitation technology and yeast two-hybrid analysis, proteins that interact with S100P can be isolated. If the selected proteins' functions are known, studying the function of these proteins will be helpful to figure out the biological function of S100P. A potential problem with this strategy is that functions of the selected proteins may be unknown. For example, S100P was found to interact with S100Z through a yeast two-hybrid analysis. S100Z was found to be a new S100 family member through this, so it is even more novel than S100P. The functions of both S100P and S100Z are unknown.

The fourth strategy is to study the expression pattern of S100P in myeloid neoplasia, specifically MDS, TDN-AML, and MDR-AML. Since S100P expression is uniquely upregulated by DNA cross-link damage, which has been associated with MDS and MDR-AML, it will be interesting to test S100P expression in healthy controls, MDS patients, and MDR-AML patients. Because of similarities in S100P and Fanconi Anemia (FA) proteins, expression of S100P in FA would also be interesting. Different S100P expression patterns

between low and high grade MDS, or between TDN-AML and MDR-AML, may be analogous to the S100P expression change after HN_2 challenge in the cell lines, and may suggest a function of S100P in the pathogenesis of MDS and MDR-AML. The expression of S100P could be detected by ICC and flow cytometry with S100P antibody.

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