The Protective Effect of Metallothionein Against Lipid Peroxidation Caused by Retinoic Acid in Human Breast Cancer Cells

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ABSTRACT
The treatment of breast cancer by retinoic acid (RA) may be mediated by lipid peroxidation. Expression of metallothionein (MT) in cancer cells, however, can protect against lipid peroxidation by scavenging hydroxyl radicals. In this study, a two-by-six factorial design was used to investigate the interactive effects of all-trans-RA and zinc (Zn)-induced MT on the growth of two human breast cancer cell lines differing in basal expression of MT and estrogen receptors; MCF7 cells express estrogen receptor, BT-20 cells do not. Cells were treated with Zn to induce MT and then treated with six RA concentrations. Cell proliferation, lipid peroxidation, MT protein, MT mRNA and glutathione concentrations were measured. BT-20 cells expressed higher constitutive MT concentrations than MCF7 cells. MT was significantly increased by Zn treatment in BT-20 cells but not in MCF7 cells. Low RA concentrations stimulated growth proliferation but higher concentrations inhibited cell proliferation. Elevated RA concentrations increased lipid peroxidation as measured by thiobarbituric acid reactive substances. There was a significant negative correlation between lipid peroxidation and cell proliferation. Growth inhibition and lipid peroxidation were reduced by Zn pretreatment in BT-20 cells but not in MCF7 cells. RA increased MT levels in both cell lines, which suggests that RA may generate free radicals which will induce MT mRNA expression. Glutathione did not appear to be a significant factor. Therefore, induction of MT by Zn may modulate the growth inhibitory effects of RA in human breast cancer cells. One mechanism of growth inhibition may be through increased lipid peroxidation. Induction of MT by RA may be one explanation for acquired RA resistance in cancer.

Suggested mechanisms of inhibition of cancer cell proliferation by RA include induction of differentiation or induction of apoptosis (Smith et al., 1992), modulation of gene expression through nuclear receptor binding, modulation of oncogene expression (Doyle et al., 1989; Hino et al., 1989; Scheibe et al., 1991) or modification of cell membrane glycoproteins (Chan and Wolf, 1987) and glycolipids (Chen et al., 1989), which could alter cell-to-cell communication, cell adhesion and shape. However, none of these mechanisms fully explain how cancer cell growth may be resistant to or stimulated by RA treatment as observed previously (Lotan, 1979). RA at low concentrations may reduce cellular oxidative stress by scavenging lipid peroxyl radicals (Samokyszyn and Marnet, 1990) and decreasing levels of superoxide (Witz et al., 1980). An increase of either oxygen free radicals or PUFA could lead to increased levels of lipid peroxidation.

One feature of rapidly proliferating cells is low levels of lipid peroxidation (Rice-Evans and Burdon, 1993) which may be caused by lower PUFA membrane content. Decreased PUFA content in cancer cells is associated with malignant transformation, tumorigenicity and metastasis (Friedberg et al., 1986). Alterations in membrane fatty acid composition may be caused by the loss or decrease of the Δ-6-desaturase activity observed in malignant tumors (Morton et al., 1979). Increasing PUFA content of cells increases sensitivity to oxygen radical toxicity (Spitz et al., 1992), inhibits cell proliferation (Perkins and Duncan, 1991) and increases lipid peroxidation (Zhang and Sevanian, 1991). Therefore, modu-
loration of cancer cell growth may be possible through manipulation of cellular membrane PUFA to increase the degree of lipid peroxidation.

One possible determinant of oxidative stress in breast cancer cells is MT. MT is a low molecular weight protein high in cysteine content that is involved in Zn homeostasis, heavy metal protection and free radical scavenging. Expression of MT can be induced by Zn and other metals, glucocorticoids, interferon, cAMP and interleukin-1, physical stresses including food restriction and extreme cold, numerous drugs, herbicides, solvents, alkylating compounds and ionizing irradiation (Dunn et al., 1987). In breast cancer, tumor overexpression of MT is associated with decreased patient survival (Fresno et al., 1993) and shorter disease-free intervals (Morales et al., 1994; Goulding et al., 1995). An inverse correlation between the expression of ER and MT has been identified (Fresno et al., 1993; Oyama et al., 1996) although not in all studies (Reid et al., 1992; Goulding et al., 1995). MT has conferred resistance to the antineoplastic drugs, cis-diaminedichloroplatinum, chlorambucil and melphanal (Kelley et al., 1988). Possible resistance to the effects of RA by MT has not been investigated.

The roles of lipid peroxidation in determining the efficacy of RA treatment in breast cancer cells have not been elucidated. It is also not known if MT can modulate the antiproliferative effects of RA. This study was designed to investigate the effects of RA on breast cancer cell proliferation in conjunction with levels of lipid peroxidation as a possible mechanism. We also examine the modulation of RA effects by different concentrations of MT present in the cells. We hypothesize that increased MT will decrease lipid peroxidation caused by RA, which will alleviate RA antiproliferative effects.

Materials and Methods

Cell culture. Human breast cancer cell lines, MCF7 (ER positive) and BT-20 (ER negative), were purchased from American Type Culture Collection (Rockville, MD). Cells were routinely propagated in Dulbecco’s Modified Essential Medium and supplemented with 10% non-heat-inactivated fetal bovine serum in humidified 5% CO2/95% air at 37°C. Zn concentration of the media was 3.3 ± 1 nM. Experiments were conducted with use of cells cultured in T75 tissue flask or 96-well plates.

Metallothionein induction with Zn. Expression of MT in both cell lines was studied after treatment with 50, 100 or 200 μM ZnCl2 for 48 h. MT was measured by a competitive ELISA (Chan et al., 1992). Total protein was measured with the Bradford assay (Bradford, 1976), so MT was standardized to nanograms per milligram of protein.

Interaction between Zn and RA. A two-by-six factorial experiment was conducted. Cells grown in both 96-well plates or T75 tissue flask were pretreated with 200 μM Zn (14 days) and then treated with RA (1 × 10−10 to 5 × 10−6 M for 7 days). RA solutions were prepared fresh in 95% ethanol. All manipulations were performed under yellow light. Total ethanol used as a vehicle for RA amounted to <0.1% (a concentration that has been shown not to affect cellular proliferation). Medium was changed once on day 4. On day 8, cell proliferation was measured. Cells were harvested with 0.29% trypsin/1 mM ethylenediaminetetraacetic acid. Aliquots of cells were used for lipid peroxidation measurement, MT measurement or glutathione measurement or treated with Trizol for RNA isolation.

Cell proliferation. The experiment was conducted with cells plated to 96-well plates. Cell proliferation was measured by the Promega CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Cory et al., 1991). A tetrazolium salt (MTS) was bioreduced by cells to a media-soluble formazan, which was measured spectrophotometrically at A570 with a V_max microplate reader (Molecular Devices Corporation, Menlo Park, CA). The formation of formazan from MTS was directly proportional to the number of cells present. All values were represented as percent of controls.

Lipid peroxidation. Harvested cells were lysed in 1.25 ml cold 154 mM potassium chloride and 5 μM butylated hydroxytoluene with a sonicator. The lysed cell suspension (0.5 ml) was added to 3 ml cold 1% (w/v) phosphoric acid. As an indication of the degree of lipid peroxidation, a TBARS assay was performed (Sunderman et al., 1985). Protein concentrations were measured by the Bradford assay (Bradford, 1976) to standardize between samples.

MT measurement. MT in the harvested cells were measured on both the protein and mRNA level. MT protein level was measured by ELISA as described by Chan et al. (1992). Total RNA was isolated with Trizol reagent (Gibco BRL, Grand Island, NY). RNA (10 μg) was denatured and subjected to electrophoreses through a 1% agarose gel followed by transfer to Hybond nylon membrane (Amersham, Arlington Heights, IL). Northern blots were hybridized to 32P-labeled human hMT-IIA genomic probe (Koropatnick et al., 1989). Autoradiography was done with X-OMAT AR Scientific Imaging Film (Kodak, Rochester, NY) followed by densitometry. Rehybridization of the same nylon membranes to an 18S-labeled probe allowed for standardized qualitative comparison between samples.

Glutathione measurement. Harvested cells were immediately resuspended in 2.5% perchloric acid and stored at −80°C until analysis. Total reduced and oxidized GSH was measured as described by Anderson (1985). Total protein was measured so all values were standardized to nanomoles GSH per milligram protein.

Statistical methods. All statistics were performed with Systat for Windows Version 5.02 (Systat Inc., Evanstown, IL). A two-way ANOVA was used for each cell line to examine the effects of Zn and RA treatments on lipid peroxidation, MT concentrations and GSH concentrations. The differences between treatment groups were determined by Tukey’s HSD Multiple Comparison Test. The Pearson product moment correlation coefficient of cell proliferation and lipid peroxidation was calculated. A significance level of P < .05 was used for all tests.

Results

Basal MT concentrations were significantly higher in BT-20 cells at 30 ± 3 ng/mg protein (n = 3) than in MCF7 cells with MT concentrations of 5 ± 2 ng/mg protein (n = 3). With 48-h treatments with 50, 100 and 200 μM Zn, there was a dose-dependent increase of MT in the BT-20 cells (ANOVA, P < .05) (fig. 1). The a posteriori test results show that BT-20 cells treated with 200 μM Zn had significantly higher MT than the other groups. The concentration of MT increased 10-fold to 300 ± 77 ng/mg protein. There was, however, no significant induction of MT in MCF7 cells.

With RA treatments there was a consistent dose-dependent growth pattern observed in both the control and Zn-treated groups for both cell lines (fig. 2, A and B). There was an initial increase of cell proliferation with lower concentrations of RA. At higher concentrations of RA there was a dose-dependent decrease of cell proliferation. Two-way ANOVA results showed significant effects for both Zn and RA treatments on cell growth in BT-20 cells. For the control cells there was a slight stimulation of cell proliferation at 10−10 M RA. At 10−8 M and 5 × 10−7 M RA, cell proliferation was inhibited to 89% and 83% of the control, respectively. Zn treatment of BT-20 cells resulted in statistically significant higher cell proliferation than the control cells (significant at
RA treatments of $10^{-8}$ M, $10^{-6}$ M, $10^{-5}$ M and $5 \times 10^{-5}$ M. Cell proliferation was stimulated in Zn-treated cells with $10^{-8}$ M and $10^{-6}$ M RA to 112% and 108% of control, respectively. On the other hand, a significant growth inhibition occurred from treatment with $5 \times 10^{-5}$ M RA, which was 91% of control.

In MCF7 cells, two-way ANOVA results showed no significant differences in cell proliferation between the control and Zn-treated cells, and no interaction between Zn and RA treatments. However, there were significant differences in cell proliferation between levels of RA treatment in MCF7 cells. Treatment with $10^{-10}$ M RA increased cell proliferation 10% in control and in Zn-treated cells, but the effect was only statistically significant in Zn-treated cells ($n = 96$). There was no effect at $10^{-8}$ M RA. At $10^{-6}$ M, $10^{-5}$ M and $5 \times 10^{-5}$ M RA, there was a significant dose-dependent inhibition of cell proliferation in both the control and Zn-treated cells.

Results from the TBARS assay showed a significant effect of RA and of Zn on lipid peroxidation in BT-20 cells (fig. 3A). There was also a significant effect of RA in MCF7 cells but, in contrast to BT-20 cells, there was no significant effect of Zn (fig. 3B). Basal levels of lipid peroxidation were 0.059 ± 0.009 mmol MDA chromogens per gram protein in BT-20 cells ($n = 3$) and 0.025 ± 0.001 mmol MDA chromogens per gram protein in MCF7 cells ($n = 3$). Significant increases of lipid peroxidation in BT-20 cells was observed with $5 \times 10^{-5}$ M RA. There were significant increases in lipid peroxidation with $10^{-5}$ M and $5 \times 10^{-5}$ M RA up to three times the basal levels in MCF7 cells.

There was a statistically significant negative correlation between cell proliferation and lipid peroxidation in both cell lines (fig. 4, A and B). The Pearson product moment correlation coefficient calculated for these measures was $R^2 = 0.765$ for BT-20 cells and $R^2 = 0.676$ for MCF7 cells.

The 14-day, 200 μM Zn treatment increased MT levels in BT-20 cells 40-fold to 1200 ± 300 ng/mg protein ($n = 3$) (fig. 5, A and B). MT was also induced in both cell lines after RA treatment (fig. 5, A–D). There was a significant MT increase in Zn-treated BT-20 cells with $10^{-10}$ and $10^{-9}$ M RA treatments. The highest concentration of MT was measured at 8000 ± 2000 ng/mg protein in cells treated with Zn and $10^{-5}$ M RA. In MCF7 cells, MT levels were significantly higher in cells treated with $5 \times 10^{-5}$ M RA ($n = 3$).

Qualitative investigation of the MT-2 mRNA levels supports MT protein measurement. Measurement of MT-2 mRNA was detectable in both control and Zn-treated BT-20 cells (fig. 6A). Levels of MT-2 mRNA were 600% to 800% higher in Zn-treated BT-20 cells that also were treated with RA. In BT-20 cells not treated with Zn, a dose-dependent effect of RA was also observed. With $5 \times 10^{-5}$ M RA treatment, MT-2 mRNA was increased to 200% of control. In MCF-7 cells, MT-2 mRNA was detected in Zn-treated cells but not in control cells (fig. 6B). MT-2 mRNA increased dose-dependently with RA treatment in Zn-treated MCF7.
cells. There was a 2.5- and 3-fold increase in Zn-treated MCF7 cells with treatments of 10^{-5} and 5 \times 10^{-5} M RA, respectively.

GSH measurement showed a significant effect of RA treatment but not of Zn treatment on GSH concentrations in BT-20 cells (fig. 7A). There were significantly lower GSH concentrations in BT-20 cells treated with 5 \times 10^{-5} M RA. In Zn-treated BT-20 cells, GSH was significantly decreased with the 5 \times 10^{-5} M RA treatment compared with 10^{-10} M RA-treated cells. In MCF7 cells, there was a significant effect of Zn treatment but not RA treatment on GSH concentrations (fig. 7B). Basal GSH concentrations measured in BT-20 cells were 70 \pm 6 nmol/mg protein (n = 3) and in MCF7 cells they were 32 \pm 7 nmol/mg protein (n = 3).

**Discussion**

The expression of MT in breast cancer has significant health implications. Increased expression of MT is associated with decreased patient survival and disease-free survival (Reid et al., 1992; Fresno et al., 1993; Morales et al., 1994; Goulding et al., 1995). Two human breast cancer cell lines that differed in their ER expression were selected for this study with the hypothesis that they would differ in their expression of MT. Measurement of basal MT concentrations in the cell lines indicated ER-negative BT-20 cells had 3-fold higher expression of MT (30 ng/mg protein) than ER-positive MCF7 cells (10 ng/mg protein). In the BT-20 cell line, there was a significant increase of MT to 300 ng/mg protein with Zn treatment (200 \mu M for 48 h), and an increase of up to 1200 ng/mg protein after Zn treatment for 14 days (200 \mu M). Conversely, MT was not increased in MCF7 cells with the 48-h Zn treatment and only slightly increased after a 14-day Zn treatment. MT mRNA measured by Northern blot analysis confirms the protein measurement. Similar low induction of MT in MCF7 cells has been measured (Kelley et al., 1988).

After treatment with cis-diaminedichloroplatinum, the MT concentration was measured at only 95 \pm 11\% of control. Therefore, because of their differences in basal MT and MT inducibility, these two cell lines may be used to investigate the possible role of MT in cancer cell proliferation.

With this two-cell line model, we demonstrated that Zn-induced MT can protect against cell growth inhibition caused by RA. Cell proliferation in Zn-treated BT-20 cells was up to 15\% higher than controls. However, it has been argued that the protective effect may not caused solely by MT, because Zn alone could be equally protective (Thomas et al., 1986). Because Zn is required for the function of many enzymes involved with translation and transcription (Christianson, 1991; Vallee, 1988), Zn treatment may allow for increased activity and increase the rate of cell proliferation. We have shown indirect evidence demonstrating that MT expression may be a more critical factor. After Zn treatment, MT concentrations in MCF7 cells (10 ng/mg protein) were more than 100-fold lower than in BT-20 cells (1200 ng/mg protein). Cell proliferation in Zn-treated MCF7 cells did not differ from controls, whereas a clear protective effect was observed for the same Zn treatment in BT-20 cells. These results indicate that it was the increased levels of MT, and not Zn, that was responsible for the modulation RA inhibition of cell proliferation. Increased expression of MT is one mechanism which has been implicated in acquired antineoplastic drug resistance (Harris and Hochhauser, 1992; Kelley et al., 1988; Lazo and Basu, 1991). Resistance to antineoplastic agents conferred by MT has also been demonstrated with cis-diaminedichloroplatinum, chlorambucil and melphalan (Kelley et al., 1988).

In this study, RA was shown to stimulate and inhibit cell proliferation in both cell lines depending on the treatment concentration. In BT-20 cells, cell proliferation was increased slightly with 10^{-10} M RA in both control and Zn-treated cells, and significantly with 10^{-8} and 10^{-6} M RA in Zn-treated cells. Inhibition of growth was observed with 10^{-5} and 5 \times 10^{-5} M RA in control cells but only with 5 \times 10^{-5} M RA in Zn-treated BT-20 cells. In both control and Zn-treated MCF7 cells, cell proliferation was stimulated with 10^{-10} M RA but inhibited with 10^{-6}, 10^{-7} and 5 \times 10^{-5} M RA. Antiproliferative effects of RA in cancer have been reported previously, although the results obtained have not been consistent in all cell types. RA used at the same concentration was found to have variable effects on growth in different cell lines (Lotan, 1979). In another study, examining the effect of atRA on the proliferation of 15 different cell lines, 9 cell lines were RA sensitive and 6 were RA resistant (Takatsuka et al., 1996). The authors attributed the differences in RA susceptibilities.
to the ability of the cells to metabolize RA. They suggested that there was a metabolite of RA affecting the cell proliferation although conditioned media from RA-metabolizing cells had no effect on the resistant cells. Another study examining the responsiveness of ovarian cancer cell lines suggested that the effectiveness of RA was related to the level of differentiation of the cell line (Caliaro et al., 1994). They found that the most undifferentiated metastatic cell line was resistant to RA. Because increased MT has been associated with a decrease in differentiation and the development of metastases (Oyama et al., 1996; Schmid et al., 1993), the intrinsic level of MT or the inducibility of MT expression may affect resistance to RA. Therefore, the variations of RA dose used and the levels of MT may explain some of the inconsistency of the effect of RA observed previously.

Our results show that the different effects of RA on cell proliferation may be explained by the modulation of cellular oxidative stress. At low concentrations, RA could act as scavenger free radicals such as fatty acid peroxyl radicals (Samokyszyn and Marnet, 1990), lowering the oxidative stress of the cell and allowing for increased proliferation. In both MCF7 and BT-20 cells, there was a slight decrease of lipid peroxidation with low doses of RA. This also corresponded with an increase of cell proliferation. This inverse relationship between cell proliferation and lipid peroxidation has been reported previously (Rice-Evans and Burdon, 1993). Conversely, increasing concentrations of RA increased lipid peroxidation, which corresponded with decreased cell proliferation. The mechanism of RA-mediated lipid peroxidation is not fully known. However, there are two possible mechanisms. RA can stimulate the activity of Δ-6-desaturase (Alam et al., 1984) resulting in an increase of polyunsaturated fatty acids, which are oxidized most easily. Δ-6-Desaturase is the enzyme responsible for inserting a double bond during PUFA synthesis. A loss or decreased activity of this enzyme has been found in some malignant tumors. RA can also directly increase free radicals (Davis et al., 1990), which could result in increased lipid peroxidation.

The observed protective effect of MT further supports that the action of RA is caused by increases of oxidative stress. MT can act as a free radical scavenger (Thornalley and Vasak, 1985) and protect against lipid peroxidation (Naganuma et al., 1988). In Zn-treated BT-20 cells, there was a decrease in lipid peroxidation compared with controls. Conversely, lipid peroxidation levels in MCF7 cells were not affected by Zn treatment. This suggests that MT is acting to decrease lipid
peroxidation, which allows for increased cell proliferation. The induction of MT by RA in breast cancer cells is a novel finding. This could be one mechanism explaining the acquired resistance in conventional RA treatments. RA is not commonly known as a MT inducer. However, 13-cis-RA has been shown to induce hepatic MT in mice and to produce a synergistic effect when combined with Zn (Cousins and Swerdel, 1985). On the other hand, elevated MT levels in mouse skin tumors were not affected by RA (Hashiba et al., 1989; Islam and Toftgard, 1992). The relationship between RA and MT induction therefore requires further investigation.

GSH is another free radical scavenger implicated in antineoplastic drug resistance (Chen et al., 1995). In control and Zn-treated BT-20 cells, there was a RA dose-dependent decrease in GSH concentrations. This decrease may be caused by GSH reacting with increased free radicals or lipid peroxides. There was no difference in GSH concentrations between zinc-treated and control BT-20 cells, showing that there is little relationship between GSH and MT levels. This suggests that GSH and MT may work through different pathways by scavenging different free radicals. Because Zn treatment had an effect on cell proliferation in BT-20 cells but GSH levels were unaffected by zinc, it is unlikely that GSH is a critical molecule in protection against RA. RA has been shown to decrease GSH concentrations in cephalic chondrocytes, although RA had no effect on GSH concentrations in immature caudal chondrocytes (Teixeira et al., 1996). Therefore, the effect of RA may depend on the maturation and stage of cell differentiation. In MCF7 cells, RA did not alter GSH, but Zn treatment had an effect. This could result from the availability of high levels of free Zn because there was no increase of MT with 200 μM Zn exposure. Zn has inhibited the activity of cytochrome c reductase (Kubow et al., 1984) which could decrease the formation of reactive species from RA. Free zinc could also displace iron from lipid membranes which could reduce free radical formation from the Fenton reaction and subsequent lipid peroxidation (Girotti et al., 1985). By reducing the formation of reactive species or lipid peroxidation, degradation of GSH may have been protected indirectly by unbound Zn. However, because there was no significant decrease in lipid peroxidation or protective effect on cell proliferation, it is unlikely that this sparing of GSH in MCF7 cells is biologically important.

Results of our experiments show that both RA and Zn can affect the growth of human breast cancer cells. Zn treatment induced MT in BT-20 cells and increased cell proliferation after RA treatment compared with controls. Although Zn concentrations used in this experiment are higher than physiological plasma concentrations (200 μM vs. 15 μM), it shows that MT can be induced in breast cancer cells. In addition to
Zn, MT can be induced by a variety of compounds and situations such as glucocorticoids, interferon, interleukin-1, stress and food restriction (Dunn et al., 1987). Because these compounds or situations may be present during cancer treatment, MT could be induced in the tumor resulting in increased resistance to treatment. Because MT does not provide protection against all cancer therapies, more effective treatments may be selected once MT expression of the cancer is determined.

Although the effects of RA on cancer are generally thought to result from interaction with nuclear receptors, our results suggest that modulation of lipid peroxidation may also be an important factor. Increasing RA increased lipid peroxidation and this was correlated with inhibition of cell proliferation. By increasing or decreasing the levels of lipid peroxidation, RA could regulate cell growth. The ability of the cell to protect itself from the increased lipid peroxidation could be an indicator for the efficacy of RA. Cells that express high levels of MT may be more resistant to its antiproliferative effects. Additionally, other oxygen free radical scavengers, such as catalase, superoxide dismutase or GSH, may affect the efficacy of RA, although GSH seemed to have little effect in this system. Levels of these scavengers may help explain how RA mediates growth in various cell lines.

The concentrations of RA used in this study range from physiological plasma concentrations to pharmacological concentrations. The results show the importance of RA concentrations for the stimulation or inhibition of cell growth. In previous experiments, $1 \times 10^{-6}$ M RA was the concentration used when cell proliferation of mammary and epithelial cell lines was either inhibited, stimulated or resistant (Lotan, 1979). Pharmacological RA doses result in transient plasma concentrations of $3 \times 10^{-6}$ M, the range at which cell growth was inhibited in MCF7 cells but not BT-20 cells, and stimulated in BT-20 cells with high MT. This study may also assist in explaining the results of the ATBC (Rautalahti et al., 1995) and CARET (Omenn et al., 1994) studies. β-Carotene can be converted directly to RA so the β-carotene supple-

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**Fig. 6.** MT-2 mRNA induction after treatment with zinc and retinoic acid. Cells were pre-treated with 200 μM Zn for 14 days, then treated with RA ($1 \times 10^{-12}$ to $5 \times 10^{-5}$ M) for 7 days. RNA was isolated from cells, denatured, electrophoresed through a 1% agarose gel, then transferred to a nylon membrane. Northern blots were hybridized to a $^{32}$P-labeled human hMT-IIA genomic probe. Bound probe was detected with and measured by densitometry. Rehybridization of the same nylon membranes to an 18S-labeled probe allowed for standardized qualitative comparison between samples.


cancer in high risk populations: smokers and asbestos-exposed workers.

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