Oxidation and Nuclear Localization of Thioredoxin-1 in Sparse Cell Cultures

Jeanine C. Spielberger, Amie D. Moody, and Walter H. Watson*

Department of Environmental Health Sciences, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland 21205

Abstract   Reactive oxygen species (ROS) were once viewed only as mediators of toxicity, but it is now recognized that they also contribute to redox signaling through oxidation of specific cysteine thiols on regulatory proteins. Cells in sparse cultures have increased ROS relative to confluent cultures, but it is not known whether protein redox states are affected under these conditions. The purpose of the present study was to determine whether culture conditions affect the redox state of thioredoxin-1 (Trx1), the protein responsible for reducing most oxidized proteins in the cytoplasm and nucleus. The results showed that Trx1 was more oxidized in sparse HeLa cell cultures than in confluent cells. The glutathione pool was also more oxidized, demonstrating that both of the major cellular redox regulating systems were affected by culture density. In addition, the total amount of Trx1 protein was lower and the subcellular distribution of Trx1 was different in sparse cells. Trx1 in sparse cultures was predominantly nuclear whereas it was predominantly cytoplasmic in confluent cultures. This localization pattern was not unique to HeLa cells as it was also observed in A549, Cos-1 and HEK293 cells. These findings demonstrate that Trx1 is subject to changes in expression, redox state and subcellular localization with changing culture density, indicating that the redox environments of the cytoplasm and the nucleus are distinct and have different requirements under different culture conditions. J. Cell. Biochem. 104: 1879–1889, 2008.
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The concept of redox signaling stems from the observation that stimulation of some receptors results in the intracellular production of reactive oxygen species (ROS) and that these ROS alter the activity of signaling molecules within the cell [Sundaresan et al., 1995; Bae et al., 1997]. Many proteins, including protein tyrosine phosphatases, kinases and transcription factors, contain reactive cysteine thiols that can be readily oxidized to sulfenic acids and disulfides by ROS. Because most intracellular proteins are reduced under basal conditions, these reversible redox reactions allow cellular proteins to sense and respond to ROS from both exogenous and endogenous sources [Finkel, 2003]. The types of responses elicited by transient oxidation reactions include changes in enzymatic activity, protein-protein interactions and protein–DNA interactions [Watson et al., 2004].

Thioredoxin-1 (Trx1) is a highly conserved protein that is responsible for restoring oxidized proteins to their reduced forms [reviewed in Powis et al., 2000]. When Trx1 reduces an oxidized substrate protein, the 2 active site cysteines of Trx1 become oxidized to a disulfide that is then reduced by thioredoxin reductase using electrons from NADPH. In cells growing under standard culture conditions greater than 90% of cellular Trx1 is in the reduced form [Nkabyo et al., 2002; Watson et al.,

2003]. During redox signaling induced by growth factors or upon exposure to oxidants, the relative amount of oxidized Trx1 in the cell increases then returns to baseline levels in a relatively short time [Watson and Jones, 2003; Hansen et al., 2004; Halvey et al., 2005], reflecting a transient increase in the rate of reaction with oxidized substrates.

An early study of the distribution of mammalian Trx1 revealed widespread expression in most calf tissues, mostly associated with cytosolic, nuclear and membrane fractions [Holmgren and Luthman, 1978]. Subsequent investigations in other species confirmed this localization pattern but also demonstrated that the relative level of Trx1 in different subcellular compartments is variable. For example, epithelial cells in the human cervix have high levels of both nuclear and cytoplasmic Trx1 whereas Trx1 in stromal cells is largely nuclear but becomes more cytoplasmic during pregnancy [Lysell et al., 2003]. In contrast, rat brain neurons Trx1 is predominantly cytoplasmic, but translocates to the nucleus following ischemia-reperfusion injury [Takagi et al., 1998]. Redistribution of Trx1 from the cytoplasm to the nucleus has also been observed in cell culture models. Exposure of cultured cells to ionizing radiation, growth factors and inflammatory cytokines results in the nuclear translocation of Trx1 [Hirota et al., 1999; Wei et al., 2000; Wiesel et al., 2000; Bai et al., 2003]. Independent studies have shown that these stimuli result in the intracellular production of ROS [Janssen et al., 1993; Higuchi et al., 2002; Galeotti et al., 2005], suggesting that ROS are involved in signaling the nuclear translocation of Trx1.

Sparse cell cultures have been shown to have elevated ROS levels [Pani et al., 2002; Bello et al., 2003]. In the current studies, we describe changes in the redox state, expression and localization of Trx1 in cell culture in response to changes in cell density. Specifically, sparse cells have more oxidized Trx1 and more nuclear Trx1 despite having less total Trx1 relative to confluent cultures.

MATERIALS AND METHODS

Cell culture. HeLa cells, A549 cells, Cos-1 cells and HEK293 cells (ATCC) were cultured at 37°C in DMEM supplemented with 10% fetal bovine serum and penicillin and streptomycin in a humidified atmosphere containing 5% CO_2. Low, medium and high density cultures were achieved by seeding 6-well plates with 40,000 cells per well, 200,000 cells per well and 400,000 cells per well, respectively, and culturing for 2 days.

Indirect immunofluorescence microscopy. For these experiments, cells were plated as described above in 6-well plates containing coverslips. Cells were fixed, permeabilized and blocked as described [Griffis et al., 2002]. Immunostaining was performed using primary antibodies specific for Trx1 (sources are noted in figure legends), and secondary antibodies conjugated to Alexafluor-488 or Alexafluor-555 (Molecular Probes), where indicated. Coverslips were mounted onto glass slides using Vectashield containing Dapi (Vector Laboratories H-1200). Images were obtained using either laser confocal (Zeiss LSM 510 META laser scanning microscope system and Zeiss LSM 5 software) or fluorescence microscopy (Nikon Eclipse TE300 microscope and SPOT camera and software from Diagnostic Instruments, Inc.).

Western blotting. Cells were lysed in RIPA Buffer consisting of $1\times$ PBS, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 0.2 mM NaVO$_4$, 0.2 μM PMSF, 1 μg/ml pepstatin, 5 μg/ml leupeptin and 5 μg/ml aprotinin. After incubation on ice for 30 min, the cell lysate was centrifuged for 10 min at 10,000g at 4°C. Protein concentration of the supernatant was determined using the Bio-Rad DC protein assay kit according to the manufacturer's instructions. Equal amounts of protein (20 μg) were separated by 15% SDS–PAGE and transferred by electroblotting to nitrocellulose membranes (Bio-Rad). Membranes were blocked in a 1:1 solution of Odyssey blocking buffer (LI-COR) and PBS, and incubated with primary antibodies against Trx1 (American Diagnostica) and β-actin (Santa Cruz) for 1 h each. Secondary antibodies were labeled with Alexafluor 680 (Molecular Probes). Membranes were scanned using Odyssey Infared scanner (LI-COR) quantitative analysis was performed using Odyssey software (LI-COR).

Redox Western blot. The redox state of Trx1 was determined as described in [Watson et al., 2003]. HeLa cells growing at low and high density were washed with cold PBS and lysed in denaturing lysis buffer containing 50 mM iodoacetic acid to carboxymethylate thiols (but not

disulfides or sulfenic acids). The redox forms of Trx1 were separated by native PAGE and visualized by western blotting as described above, using anti-human Trx1 antibody from American Diagnostica.

Measurement of GSH and GSSG. S-Carboxymethyl N-dansyl derivatives of the reduced and oxidized forms of glutathione were separated by HPLC and detected by fluorescence, as described [Jones, 2002].

DCF fluorescence assay. Low and high density cultures were incubated for 30 min with 20 μM 2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA; Invitrogen), trypsinized and resuspended in cold PBS. Fluorescence intensity of DCF was by measured by FACScan and analyzed with BD CellQuest software (Becton Dickinson).

Thioredoxin reductase activity assay. Cells were plated at low and high density, cultured for 48 h and lysed in 10 mM Tris, pH 7.5, 0.5% (v/v) Triton X-100, 0.5% (w/v) deoxycholate, 0.1% (w/v) SDS, 150 mM NaCl, 1mM EDTA, with freshly added pepstatin, leupeptin, aprotinin and PMSF for 30 min on ice [Fang et al., 2005]. Lysates were cleared by centrifugation for 15 min at 15,000g at 4°C and total protein was determined by the Bio-Rad DC method (Bio-Rad) using gamma-globulin as the standard. Thioredoxin reductase activity was determined in cleared supernatants according to previously published methods [Arner and Holmgren, 2005]. In a total volume of 50 μl, 20 μg of supernatant protein was incubated with 200 μM oxidized insulin and 440 μM NADPH in 55 mM Hepes, pH 7.6, and 2.7 μM purified recombinant human Trx1 (American Diagnostica). A duplicate reaction containing everything except recombinant Trx1 was used as the blank. After incubation for 1 h at 37°C, total thiols in the two paired reactions were measured by adding 500 μl of 1 mM DTNB in 6 M guanidinium and measuring the absorbance at 412 nm. The absorbance from the blank sample was subtracted from the absorbance obtained by the addition of Trx1. The moles of TNB formed were calculated from the molar extinction coefficient ($13,600$ M^{-1} cm^{-1}) and the activity was expressed as μmol TNB formed per mg protein per hour (mean ± SD).

Statistical analyses. Results were analyzed by ANOVA followed by Student's two sample t-test. Results were considered statistically significant if $P < 0.05$.

RESULTS

Trx1 Is More Oxidized in Sparse Cultures Than in Confluent Cultures

Trx1 is maintained largely in the reduced form through the action of thioredoxin reductase, but exposure of cells to growth factors or oxidants results in the transient accumulation of the oxidized form [Watson et al., 2003; Halvey et al., 2005]. To determine whether oxidative stress associated with sparse culture conditions affected the thioredoxin system, the redox state of Trx1 was measured in low and high density cultures using the Redox Western blot. In this method, the oxidized and reduced forms are separated by native PAGE after derivatization of the thiols with iodoacetic acid. The results show that the proportion of Trx1 in the oxidized form was greater in sparse (low density; 20% confluent) cultures than in confluent (high density; 95% confluent) cultures (Fig. 1A). In the high density cultures only 10% of the Trx1 was in the oxidized form, but in the low density cultures 26% was oxidized (Fig. 1B).

The redox state of Trx1 reflects the balance between two opposing reactions: oxidation of the active site through reduction of substrates and reduction of the active site through reaction with thioredoxin reductase. One possible explanation for the oxidation of Trx1 in low density cultures is that these cells have less thioredoxin reductase activity. However, this does not appear to be the case. The activity of thioredoxin reductase in lysates from low density cells is actually greater than the activity in high density cells: 2.47 ± 0.62 versus 1.60 ± 0.21 μmol/mg protein/h ($P < 0.005$, n = 8). Therefore, it is not likely that differences in thioredoxin reductase activity account for the observed differences in Trx1 redox state.

If the Trx1 in low density cells is oxidized because of an increase in the number of substrates, then this might be reflected in an oxidation of glutathione, the other major redox regulating system. The amount of reduced glutathione (GSH) was the same in both low and high density cultures, but the amount of oxidized glutathione (GSSG) was significantly higher in low density cells (Fig. 2). ROS generation (primarily H$_2$O$_2$) was determined in low and high density cells by incubating cells with the ROS-sensitive probe H$_2$DCFDA for 30 min. Fluorescence intensity resulting from oxidation of this probe was 2.6-fold greater in
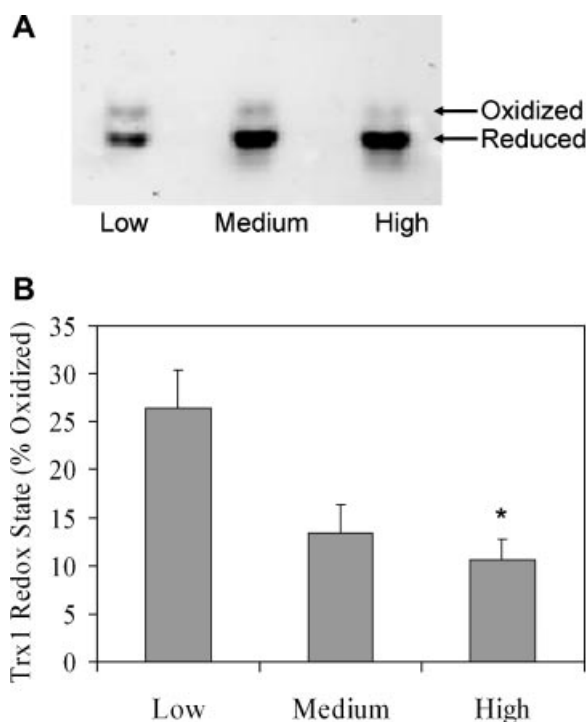
A

Low   Medium   High

B

Fig. 1. Trx1 is oxidized in low density cultures. **A**: The redox state of Trx1 in low, medium and high density cultures was determined by Redox Western blot analysis in which oxidized and reduced forms of Trx1 are separated by native polyacrylamide gel electrophoresis. A representative Redox Western blot is shown. The upper band is the oxidized form and the lower band is the reduced form of Trx1. **B**: The experiment shown in part (A) was repeated five times and the relative intensities of the bands corresponding to the oxidized and reduced forms were determined using LiCor Odyssey software. The data represent the means ± SEM of five separate measurements. The asterisk indicates $P < 0.05$ relative to low density cultures.
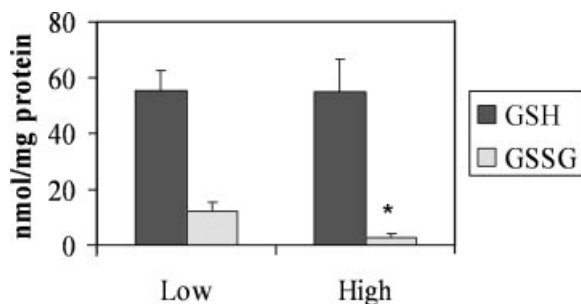

Fig. 2. Redox state of GSH/GSSG in low and high density cultures. GSH and GSSG levels were determined in low and high density cells by HPLC. The gray bars are the mean GSH values and the hatched bars are the mean GSSG values for three independent measurements. The error bars represent the SEM and the asterisk indicates a significant difference ($P < 0.05$) from the corresponding value in low density cells.
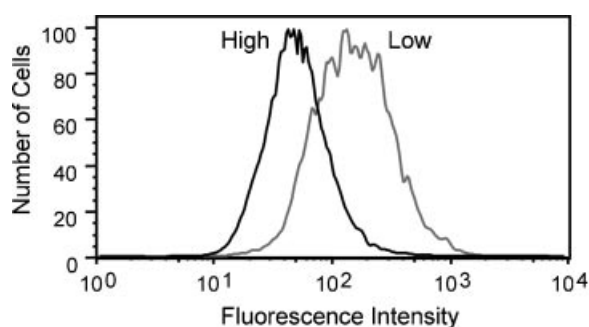

Fig. 3. ROS levels are elevated in low density cultures. High and low density HeLa cells were incubated with 20 µM H_2DCFDA for 30 min. The amount of DCF produced was measured by FACS analysis of the resulting fluorescence. The experiment shown was repeated 3 times.

low density cells than in high density cells (Fig. 3), indicating that ROS are being produced at a greater rate in sparse cultures than in confluent cultures. Therefore, our results confirm earlier reports that sparse cultures have elevated ROS [Pani et al., 2002; Bello et al., 2003] and suggest that the oxidation of Trx1 results from an increased rate of oxidation rather than a decreased rate of reduction by thioredoxin reductase.

Trx1 Protein Levels Increase With Increasing Cell Density

The Redox Western blot data indicated that the total amount of Trx1 increased with increasing cell density (Fig. 1A). This observation was verified by traditional western blotting. The amount of Trx1 increased with increasing cell density while the expression level of β-actin did not change (Fig. 4). Densitometric analysis of three separate experiments performed in duplicate revealed that the ratio of Trx1 to β-actin was 2.4-fold higher in high density cells than in low density cells (2.2 ± 0.062 (n = 3) vs. 0.9 ± 0.27 (n = 3), $P = 0.02$).
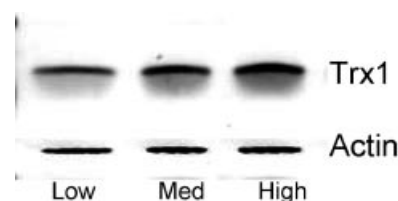

Fig. 4. Trx1 expression increases with increasing cell density. Whole cell lysates were prepared from cells plated at low, medium and high density and cultured for 2 days. Equal amounts of total protein (20 µg) were analyzed for Trx1 and β-actin protein levels by western blotting. The western blot shown is representative of three separate experiments.

Culture Density Affects Trx1 Localization

Low density cells exhibited a predominantly nuclear Trx1 staining pattern using fluorescence microscopy, whereas Trx1 staining in high density cells was mostly cytoplasmic (Fig. 5A). In cells growing at an intermediate density Trx1 was evenly distributed between both compartments (not shown). Similar results were obtained using a total of three different antibodies (two monoclonal and one polyclonal) from different commercial sources (Fig. 5A). To confirm the findings shown in Figure 5A, laser confocal microscopy was used to visualize slices of the cells at different focal distances above the coverslip to which they were attached (Fig. 5B). The results of the laser confocal and the fluorescence microscopy were essentially the same; Trx1 was concentrated in the nuclei of low density cells and in the cytoplasm of high density cells (Fig. 5B).

The same Trx1 distribution pattern was observed in three other cell lines growing at low and high density. A549 cells are a human lung carcinoma cell line of epithelial origin, HEK293 are human embryonic kidney fibroblasts and Cos-1 cells are monkey kidney epithelial cells. Each of these cell lines, like the human cervical carcinoma-derived HeLa cells shown in Figure 5, shows predominantly nuclear staining for Trx1
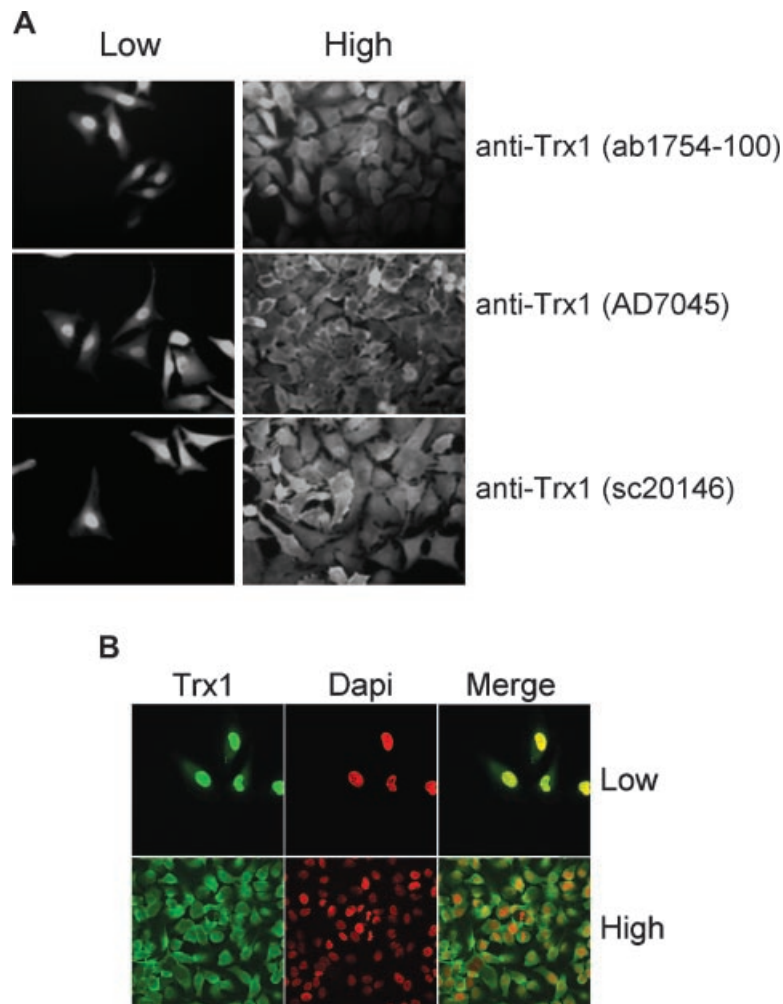

Fig. 5. Subcellular localization of Trx1 in HeLa cells cultured at different densities. **A**: Cells were plated at low and high densities in 6-well plates containing coverslips. After 2 days in culture, Trx1 was stained by indirect immunofluorescence and visualized by fluorescence microscopy. Three different antibodies against Trx1 were used (shown from top to bottom; mouse monoclonal from Abcam, mouse monoclonal from American Diagnostica and rabbit polyclonal from Santa Cruz). Nuclei were counter-stained with Dapi (not shown). **B**: Cells plated at low and high densities were stained with the polyclonal Trx1 antibody from American Diagnostica as in (A) and visualized by laser confocal microscopy. Trx1 is shown in green, Dapi is shown in red and the merged images on the right show both Trx1 and Dapi, with areas of overlapping staining shown in yellow. All images are representative of at least three separate experiments.

when grown at low density and predominantly cytoplasmic staining at high density (Fig. 6 and Table I). Therefore, the relationship between culture density and Trx1 localization is not unique to one particular cell line.

When 70% confluent HeLa cell cultures were trypsinized and re-plated at different densities, the Trx1 localization pattern rapidly changed to reflect the new culture conditions (Fig. 7). Higher numbers of cells were plated than in the previous experiments so the cultures would contain similar numbers of cells after 2 days (Figs. 5 and 6) and 2 h (Fig. 7) in culture. In cells re-plated at low density Trx1 was predominantly nuclear, while in cells re-plated at high density Trx1 was predominantly cytoplasmic 2 h after re-plating (Table II). Because these cells were pelleted and resuspended in fresh medium before re-plating, the contribution of factors released into the conditioned medium was minimized.

TABLE I. Subcellular Localization of Trx1 in Three Cell Lines

Cell line	Density	Nuc > Cyt[a]	Nuc = Cyt	Nuc < Cyt
A549	Low	71	20	9
A549	High	7	26	67
Cos-1	Low	85	15	0
Cos-1	High	7	27	66
HEK 293	Low	77	23	0
HEK 293	High	2	64	34

[a]Trx1 localization was determined by indirect immunofluorescence microscopy, as shown in Figure 6. Each individual cell was assigned to one of three categories: Nuc > Cyt, Trx1 staining predominately nuclear; Nuc = Cyt, Trx1 staining evenly distributed between the nucleus and cytoplasm; Nuc < Cyt, Trx1 staining predominately cytoplasmic. The numbers represent the percentage of the total cells in each category from multiple microscopic images.

DISCUSSION

This study demonstrates that cell culture density affects the redox balance of the cell through altered ROS production and changes in
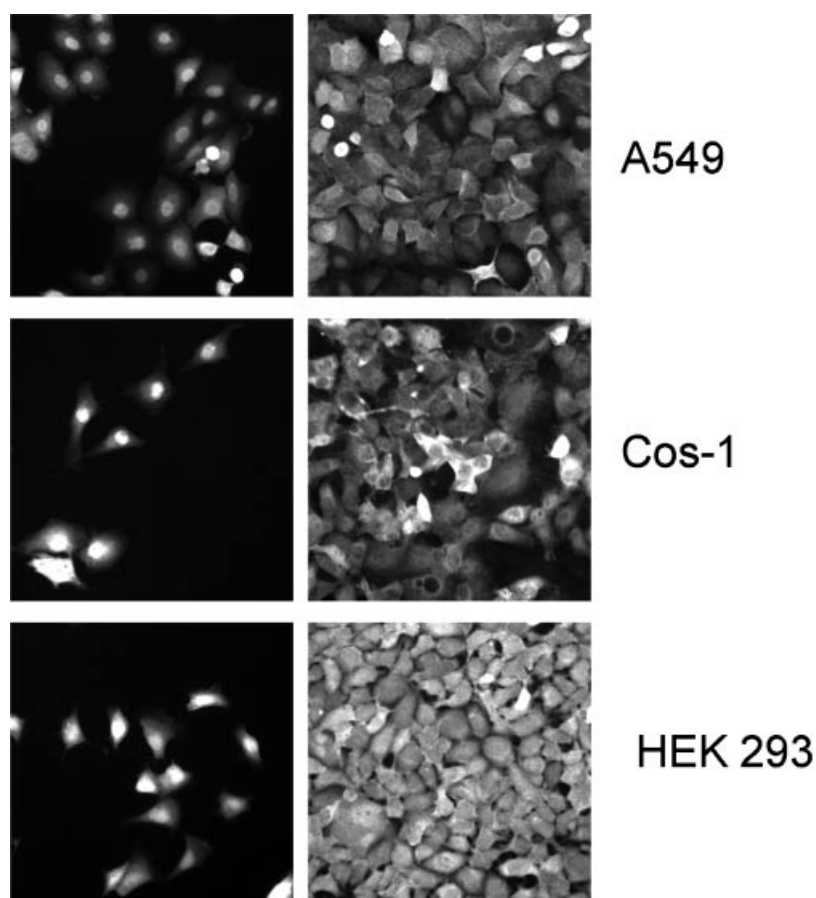

Fig. 6. Culture density affects the localization of Trx1 in other cell lines. Each of the indicated cell lines was plated in 6-well plates containing glass coverslips at low and high density, incubated for 2 days and assayed for Trx1 localization by indirect immunofluorescence microscopy using the antibody from American Diagnostica.
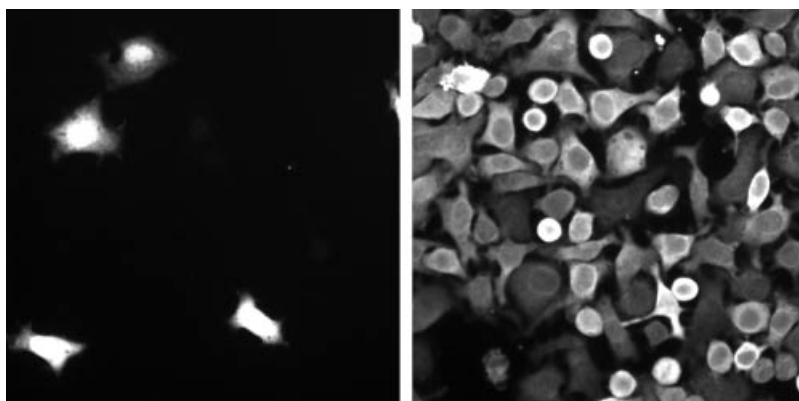
Fig. 7. Trx1 rapidly redistributes following re-plating at different densities. HeLa cells cultures at about 70% confluence were trypsinized, pelleted by centrifugation, resuspended in fresh complete medium and counted. Cells were plated at low (170,000 cells per well) and high density (1,400,000 cells per well) in 6-well plates containing polylysine-coated glass coverslips and allowed to attach for 2 h at 37°C. Coverslips were then processed for immunofluorescence microscopy using the antibody from American Diagnostica.

Trx1 redox state, protein levels and subcellular localization. Our observation that ROS levels were higher in sparse HeLa cell cultures than in confluent cultures is consistent with earlier reports that showed increased ROS under sparse culture conditions in human and mouse fibroblast cell lines [Pani et al., 2002] and in HeLa cells [Bello et al., 2003]. All of these cell lines are immortal, and it is unknown whether density-dependent ROS production is also a characteristic of primary cell cultures. In the present studies we showed not only that ROS production was increased, but also that gluta-thione and Trx1 were oxidized in sparse cells relative to confluent cells. These results demonstrate that the oxidative changes in sparse cultures are of sufficient magnitude to oxidize both of the major regulators of the thiol-disulfide redox balance of the cell.

ROS are believed to serve as mitogenic signals, probably through oxidation of cysteine residues within key regulatory proteins [Sundaresan et al., 1995]. Previous studies

TABLE II. Subcellular Localization of Trx1 2 h After Replating HeLa Cells

Density	Nuc > Cyt[a]	Nuc = Cyt	Nuc < Cyt
Low	72	28	0
High	1	26	73

[a]Trx1 localization was determined by indirect immunofluorescence microscopy, as shown in Figure 7. Each individual cell was assigned to one of three categories: Nuc > Cyt, Trx1 staining predominately nuclear; Nuc = Cyt, Trx1 staining evenly distributed between the nucleus and cytoplasm; Nuc < Cyt, Trx1 staining predominately cytoplasmic. The numbers represent the percentage of the total cells in each category from multiple microscopic images.

showed that low molecular weight protein tyrosine phosphatase is inactivated through oxidation of its catalytic cysteines in proliferating cells but becomes reduced and active during contact-dependent growth arrest [Fiaschi et al., 2001]. Redox regulation of proliferation and growth arrest almost certainly involves the coordinated activation and inhibition of multiple proteins, and the redox state of Trx1 provides a good measurement of the overall redox state of cellular proteins [Schafer and Buettner, 2001; Jones, 2002]. Because Trx1 is directly responsible for reducing protein disulfides and sulfenic acids, the Trx1 redox state is only one step removed for the redox state of other regulatory proteins. The change in the redox state of Trx1 in the absence of a change in thioredoxin reductase activity reported here is a good indication that sparse cell cultures have elevated levels of oxidized protein substrates.

Recently, we measured the redox states of glutathione and Trx1 in sparsely plated Caco-2 cells as they proliferated, reached confluence and spontaneously differentiated [Nkabyo et al., 2002]. In that study, the glutathione redox state was more oxidized in differentiated cells than in proliferating cells, primarily due to lower GSH levels. The decrease in GSH coincided with the cessation of proliferation, which occurred several days after the cells became visually confluent. In the experiments reported here, the confluent cultures were still proliferating as evidenced by BrdU incorporation, although at a reduced rate compared to sparse cultures (data not shown). HeLa cells do not spontaneously differentiate or stop dividing

upon reaching confluence, so it is unclear whether GSH levels would have decreased if the cultures had been allowed to continue past confluence. We also measured Trx1 redox state as part of the study of Caco-2 cell differentiation and found no change under any of the culture conditions. However, the total amount of Trx1 protein did increase with increasing confluence [Nkabyo et al., 2002], similar to our findings in HeLa cells.

Stress response proteins other than Trx1that have been shown to increase as cells reach confluence include MnSOD [Oberley et al., 1995], NQO1 [Bello et al., 2001], heat shock protein-27 [Garrido et al., 1997] and NADPH cytochrome b5 reductase [Bello et al., 2003]. The increase in NADPH cytochrome b5 reductase in confluent HeLa cells correlated with a decrease in levels of peroxides and superoxide anion [Bello et al., 2003] and it has been proposed that the increases in other antioxidant enzymes also contribute to the decrease in oxidative stress in confluent cells. The increased levels of Trx1 in confluent cells may be particularly relevant to the decrease in ROS because Trx1 provides reducing equivalents to the peroxiredoxins which scavenge peroxides. In support of this protective role, Trx1 overexpression has been shown to confer resistance to the toxicity of ROS [Tanaka et al., 1997; Didier et al., 2001; Shioji et al., 2002].

Although cells growing in culture are very different from cells growing within the complex matrix of a tissue, there are features of our model that may be found in vivo. Cells in sparse cultures proliferate more rapidly than cells in confluent cultures and have increased ROS levels. This combination of factors is observed during wound repair. In a mouse skin wound model, elevated levels of ROS were observed in both the extracellular fluid at the wound site and within cells at the wound edge, and treatments that decreased ROS delayed wound healing by inhibiting the proliferation and migration of cells into the wound area [Roy et al., 2006]. In the vascular system, wound repair of the endothelial cell lining contributes to atherosclerotic plaque formation. Recently, Duval et al. [2003] examined the role of ROS in the proliferation and migration of vascular smooth muscle cells and endothelial cells in an in vitro model of the repair process. This study found that both cells in sparse cultures and postconfluent monolayers that had been wounded by scraping sections of cells generated elevated levels of ROS, and these ROS were associated with increased rates of proliferation and migration [Duval et al., 2003]. Although this study focused mainly on extracellular ROS, intracellular ROS were assessed by microscopic examination of DCF fluorescence in the wounded cultures; only cells at the edge of the wound and cells that had migrated into the wound were producing elevated ROS [Duval et al., 2003]. This particular experiment was performed on ECV-304 cells, a cancer cell line [Brown et al., 2000]. Cancer cells spontaneously produce increased ROS [Szatrowski and Nathan, 1991; Hileman et al., 2004; Yen et al., 2005]. However, ROS production appears to be subject to change in response to changing conditions; sparse culture conditions resulting from either plating density (present study) or scraping a monolayer [Duval et al., 2003] promoted ROS generation and proliferation.

We found that sparse cells have more nuclear Trx1 and increased thioredoxin reductase activity relative to confluent cells. Recently, Karimpour et al. [2002] presented evidence that overexpression of thioredoxin reductase is sufficient to cause nuclear translocation of Trx1. Further experiments will be necessary to determine whether thioredoxin reductase activity contributes directly to the nuclear localization of Trx1 in sparse cells. Other proteins have been shown to move from the nucleus to cytoplasm as cells become more confluent. The aryl hydrocarbon receptor (AhR) and von Hippel-Lindau factor (VHL) shuttle between the nucleus and cytoplasm and accumulate in the nucleus in sparse cells [Lee et al., 1996; Ikuta et al., 2004], probably through inhibition of nuclear export rather than stimulation of import [Lee et al., 1999; Groulx et al., 2000; Ikuta et al., 2004]. Protein kinase C epsilon [England and Rumsby, 2000], protein tyrosine phosphatase Pez [Wadham et al., 2000] and inhibitor-2 of type 1 protein phosphatase [Leach et al., 2002] all move from the nucleus to the cytoplasm as cells reach confluence, suggesting a role for compartmentalized signal transduction in regulating proliferation or contact inhibition. Because Trx1 affects the redox state and activity of certain kinases and phosphatases [Lee et al., 1998; Saitoh et al., 1998; Kamata et al., 2005], it will be interesting to determine how cell density dependent

compartmentalization of Trx1 affects signal transduction processes.

Compartmentalization of Trx1 between the nucleus and the cytoplasm may serve multiple functions. In addition to the possible effects on phosphorylation cascades discussed above, there is increasing evidence for redox regulation of transcription factor activity within the nucleus. For example, the DNA binding activities of AP-1 [Abate et al., 1990], NF-κB [Matthews et al., 1992] and Nrf-2 [Kim et al., 2003] are inhibited by oxidation and enhanced in cells overexpressing nuclear-targeted Trx1 constructs [Hirota et al., 1997, 1999; Hansen et al., 2004]. The increase in nuclear Trx1 in sparse cells may reflect an increased demand for reducing equivalents from Trx1 for the activity of these redox-sensitive transcription factors or for the synthesis of deoxyribonucleotides for DNA replication or repair [Liu et al., 2005]. Trx1 may also be in the nuclei of proliferating cells to protect against oxidative damage to DNA and proteins through its role as a cofactor for peroxiredoxins and methionine sulfoxide reductase [Lowther et al., 2000; Banmeyer et al., 2004].

The data presented here demonstrate that sparse cultures have increased levels of oxidized Trx1 and oxidized glutathione, indicating that these cells are either under oxidative stress or are undergoing redox signaling. The changes in expression and subcellular localization of Trx1 between sparse and confluent cells suggest that the nuclear and cytoplasmic compartments represent distinct redox environments that have different requirements under different culture conditions.

ACKNOWLEDGMENTS

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