Differential Sensitivity of Human Fibroblasts and Endothelial Cells to Reactive Oxygen Metabolites

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ABSTRACT. Reactive oxygen metabolites are implicated in tissue damage, which is often followed by fibrosis. Our aim was to evaluate the sensitivity of human fibroblasts, in comparison with umbilical vein endothelial cells, to two common reactive oxygen metabolites, to superoxide produced by hypoxanthine and xanthine oxidase, and to reagent hydrogen peroxide. Depletion of the prelabeled adenine nucleotide pool, which is a sensitive index of cell damage, was used as the basis for comparison. In the presence of hypoxanthine, xanthine oxidase caused a dose-dependent nucleotide depletion, which was more pronounced in endothelial cells. After 4 h of exposure to 100 μ M hypoxanthine and 80 mU/mL xanthine oxidase, fibroblasts retained $73 \pm 2\%$ of their adenine nucleotides but endothelial cells retained only $11 \pm 2\%$. Hydrogen peroxide also had a larger effect on endothelial cells; after exposure to 100 μ M for 30 min, adenine nucleotides retained $36 \pm 26\%$ of their initial radioactivity in endothelial cells but $76 \pm 8\%$ in fibroblasts. We conclude that umbilical vein endothelial cells are inherently more sensitive to the harmful effects of reactive oxygen metabolites than are fetal skin fibroblasts. (*Pediatr Res* 32: 654–657, 1992)

Abbreviations

ROM, reactive oxygen metabolites
XO, xanthine oxidase

ROM have been implicated in cellular damage under many circumstances, for example, ischemia-reperfusion injury (1), hyperoxia (2), and inflammation (3, 4). In ischemia-perfusion injury, endothelial cells are the first target because of their localization in the vascular wall, and the first signs of damage have been demonstrated in these cells (5, 6). In chronic exposure to hyperoxia, production of ROM increases (7), and the pulmonary vascular bed may gradually disappear and be replaced by fibrosis (8, 9). ROM-induced diseases are frequent, especially in the neonatal period (10, 11).

Recently we have shown that when cultured human endothelial cells are exposed to XO (EC 1.1.3.22) and hypoxanthine, cellular adenine nucleotides are depleted (12). ATP depletion is a more sensitive indicator of cell damage than, for example, morphologic criteria or the release of radioactive chromium (13, 14), and the damage may be reversible. In the present study, our aim was to use this indicator to explore possible differences between human fibroblasts and umbilical vein endothelial cells in their sensitivity to extracellular oxidants, generated at concentrations relevant to human pathology.

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MATERIALS AND METHODS

Cell culture and labeling of adenine nucleotides. Fibroblasts derived from mid-trimester human fetal skin samples or amniotic fluid samples were cultured in Ham's F-10 medium (Gibco Europe, Paisley, UK) containing penicillin (100 U/mL), streptomycin (100 μ g/mL, Gibco), L-glutamine (2 mM, Orion Diagnostica, Espoo, Finland), and 10% FCS (Gibco). The identity of the fibroblasts was confirmed by morphologic appearance. For experiments, cells were transferred to multiwell dishes (Nunc, Roskilde, Denmark) and grown to confluency.

Human umbilical vein endothelial cells were cultured by the method of Jaffe *et al.* (15) with modifications previously described (12). They were identified as endothelial cells by the typical cobblestone pattern in phase contrast microscopy, and by the presence of factor VIII antigen assessed by indirect immunofluorescence; over 90% of the cells stained positively. For experiments, cells were grown to confluency in gelatin-coated multiwell dishes in F-10 medium with serum.

Both fibroblasts and endothelial cells were prelabeled for 16–18 h with 14 C-adenine (sp act 51–55 mCi/mmol; Amersham International, Amersham, UK), final concentration 0.1 mM, in F-10 medium with serum. The cells were then washed three times with purine-free RPMI 1640 medium (Gibco) without serum. The cells accumulate radioactivity mainly in adenine nucleotides; an average of 50% of the ATP pool is labeled (12, 16). Previously we have shown that there is no difference between the metabolism of 14 C-labeled and unlabeled nucleotides in cultured cells (17, unpublished observations).

Oxidant exposure. After prelabeling and washing the plates, RPMI 1640 medium was added to each well followed by XO (Boehringer Mannheim, Penzberg, Germany) and hypoxanthine (Sigma Chemical Co., St. Louis, MO) at the concentrations indicated. To counteract the known proteolytic activity in XO preparations (18), 5 mg/mL soybean trypsin inhibitor (Sigma) was also present in the medium. Hydrogen peroxide (Merck AG, Darmstadt, Germany) was added to final concentrations of 20 or 100 μ M.

After incubations for up to 4 h, the medium was removed and frozen. The cells were extracted twice with 0.42 N perchloric acid, and the combined extracts were stored at -20°C until analysis.

Analysis of purine compounds. Aliquots of the culture media were spotted on cellulose thin-layer chromatogram sheets (Eastman Kodak Company, Rochester, NY), and the radioactivities in total nucleotides, adenosine, inosine, hypoxanthine, xanthine, and uric acid were measured as described (12) with butanol:methanol:water:ammonia (60:20:20:1) as solvent. Cell extracts were neutralized and the purine nucleotides were separated by thin-layer chromatography on polyethyleneimine cellulose (Macherey-Nagel, Düren, Germany) using a formate gradient, then the radioactivities in the purine nucleotides were determined with a liquid scintillation counter (12). In some experiments, the radioactivities in the acid-insoluble nucleic acid fractions were also counted.

ATP concentrations in neutralized perchloric acid cell extracts were measured also with liquid chromatography, using a method modified from that described by Stocchi *et al.* (19, 20). A Shimadzu LC 6A liquid chromatograph was used with a reversed phase column (Ultra Techsphere C18, HPLC Technology Ltd., Macclesfield, UK) and UV detector set at 254 nm. Buffer A (0.1 M KH_2PO_4 , pH 6.0, containing 8 mM tetrabutylammonium hydrogen sulfate) was run at 1.5 mL/min for 2.5 min, followed by a linear increase over 12.5 min to 100% buffer B (buffer A with 30% of methanol), and continued for 5 min. ATP was identified and quantified by the retention time and peak area compared with the known standard, calibrated by spectrophotometry.

Expression of results and statistical methods. As previously shown for endothelial cells (12), radioactivity in nucleic acids or in guanine nucleotides in fibroblasts labeled with ^{14}C -adenine is small (less than 10% of total cpm incorporated) and does not change significantly upon exposure to ROM. Therefore, the results are expressed as percentage of distribution of radioactivity between the perchloric acid-soluble compounds of interest in cells (adenine nucleotides) and medium (catabolic products: hypoxanthine, xanthine, uric acid; and nucleotides).

Protein concentration was measured spectrophotometrically with the micromethod of Bradford (21) using a commercial kit (Bio-Rad Laboratories, Richmond, CA).

All experiments were performed in duplicate at least twice. Comparisons between two groups were performed by the non-parametric Mann-Whitney U-test. Results are expressed as means $\pm$ SD.

RESULTS

Effect of exogenous superoxide. When fibroblasts were incubated in serum-free medium with 80 mU/mL XO and 100 μM hypoxanthine for 4 h, cellular nucleotide levels decreased to $73 \pm 2\%$ of initial cpm, compared with $85 \pm 4\%$ in control cells (Fig. 1). There was an equivalent increase in catabolic products (hypoxanthine, xanthine, and uric acid) in the medium but no detectable release of intact nucleotides. Endothelial cells had a slightly higher basal adenine nucleotide turnover in serum-free medium, as shown by the proportion of radioactivity in nucleotides *versus* catabolic products in control cells (Figs. 1 and 3).

Fig. 1. Adenine nucleotide depletion from fibroblasts and endothelial cells prelabeled with ^{14}C -adenine, washed, and incubated with hypoxanthine (Hx, 100 μM), XO (80 mU/mL), and 5 mg/mL soybean trypsin inhibitor in serum-free medium for 4 h. Open bars indicate cellular adenine nucleotides; hatched bars indicate the sum of catabolic products (hypoxanthine, xanthine, uric acid) in the medium; cross-hatched bars indicate the sum of nucleotides in the medium. Results are expressed as percentage of total soluble radioactivity in cells plus medium (means $\pm$ SD). Asterisks indicate $p < 0.05$ compared with control (Mann-Whitney U test, $n = 4$).

There was also a small but consistent release of labeled nucleotides, which was not seen in fibroblasts. After exposure to XO and hypoxanthine, endothelial cell adenine nucleotides were nearly totally depleted ($11 \pm 2\%$ of total cpm) and a corresponding increase in catabolic products was seen ($84 \pm 1\%$ of total cpm) (Fig. 1). ATP concentrations in fibroblasts, analyzed with HPLC, showed a decrease of 19% compared with control cells (18 ± 4 nmol ATP/mg protein), which is similar to the decrease of radioactive ATP (Fig. 2). Endothelial cell ATP concentrations decreased by 81% under these conditions (Fig. 2), and control cells contained 8 ± 1 nmol ATP/mg protein.

To explore the dose-response to XO, cells were incubated with increasing concentrations of XO and 100 μM hypoxanthine. The difference in adenine nucleotide depletion between fibroblasts and endothelial cells became significant at the concentration of 40 mU/mL of XO (Fig. 3).

Fig. 2. Decrease in total ATP concentrations, measured with liquid chromatography (open bars), or in radioactive ATP, counted after separation of nucleotides with thin-layer chromatography (gray bars), in fibroblasts and endothelial cells after incubation of prelabeled (^{14}C -adenine) cells with hypoxanthine (100 μM), XO (80 mU/mL), and 5 mg/mL soybean trypsin inhibitor in serum-free medium for 4 h. Results are expressed as percentage of control cell ATP concentrations (means $\pm$ SD). For control values, see Results. Asterisks indicate $p < 0.05$ compared with control (Mann-Whitney U test, $n = 4$).

Fig. 3. Nucleotide depletion from prelabeled (^{14}C -adenine) fibroblasts (solid lines) and endothelial cells (dashed lines) incubated with varying concentrations of XO in the presence of 100 μM hypoxanthine and 5 mg/mL soybean trypsin inhibitor in serum-free medium for 1 h. Circles indicate the sum of adenine nucleotides in the cells; triangles indicate the sum of catabolic products in the medium. Results are expressed as percentage of total soluble radioactivity in cells plus medium (means $\pm$ SD). Asterisks indicate $p < 0.05$ (Mann-Whitney U test, $n = 4$) comparing the change in relation to basal nucleotide turnover (0 mU/mL XO) between fibroblasts and endothelial cells.

Effect of hydrogen peroxide. After incubation for 30 min without additions, adenine nucleotides retained $92 \pm 4\%$ of total radioactivity in fibroblasts and $80 \pm 4\%$ in endothelial cells. Hydrogen peroxide at a concentration of $20 \mu\text{M}$ had no effect on fibroblasts but caused significant nucleotide depletion after 30 min in endothelial cells (Fig. 4). At $100 \mu\text{M}$ hydrogen peroxide after 30 min, there was a decrease to $76 \pm 8\%$ of total cpm in radioactive adenine nucleotides in fibroblasts but a decrease to $36 \pm 26\%$ in endothelial cells (Fig. 5).

DISCUSSION

We have previously shown that when human umbilical vein endothelial cells are exposed to XO and hypoxanthine, their adenine nucleotides are depleted (12). This is due partly to leakage of nucleotides through the cell membrane and partly to intracellular catabolism (20). Nucleotide depletion is a sensitive indicator of cell damage (13, 14), which is not necessarily irreversible. Our experimental model is relevant to human pathophysiologic situations in which ROM are generated extracellularly, e.g. in inflammation by phagocytic cells or in ischemia-reperfusion injury, when both hypoxanthine (22) and XO (23) appear in plasma in concentrations similar to those used in our experiments.

It is not unexpected that fibroblasts *in vitro* tolerate ROM better than endothelial cells, because *in vivo* the former replace the latter in tissues damaged by ischemia-reperfusion. Several studies in intact animals and cultured cells have demonstrated

this superior tolerance, although less sensitive methods to indicate cell injury have been used (24, 25). In ischemia-perfusion injury *in vivo*, endothelial cells could be more susceptible because of their location in the front line, whereas fibroblasts would remain viable because they are further removed from the vascular wall. Our data indicate that the differential susceptibility is not due to spatial factors but is inherent to the cells.

Our results suggest that both the superoxide radical, generated by XO acting on hypoxanthine, and hydrogen peroxide have a more profound effect on cellular nucleotides in umbilical vein endothelial cells compared with fetal skin fibroblasts. In comparing the effects of XO and hypoxanthine on the two cell types, it seems that there is a release of intact nucleotides from endothelial cells but not from fibroblasts (Fig. 1). However, when hydrogen peroxide was present in the medium, there was no significant release of intact nucleotides from either cell type. Even though nucleotide efflux might, to some extent, be masked by the action of ecto-5'-nucleotidases (26), it is more likely that the depletion of adenine nucleotides is due to intracellular catabolism (27). We have previously shown that when ecto-5'-nucleotidase is inhibited, superoxide causes leakage of nucleotides in endothelial cells, indicating membrane damage, but hydrogen peroxide does not (20). The present results suggest that fibroblasts are even less prone to membrane damage.

Our aim was not to explore the mechanisms of the greater tolerance of fibroblasts to damage by ROM. Several possibilities exist, including differences in membrane structure or in endogenous antioxidant defense mechanisms (25, 28). *In vivo*, endothelial cells have been shown to suffer morphologic damage early in the course of tissue injury (5, 29, 30). The more durable fibroblasts would remain undamaged and be able to proliferate in the reparative stage of the process, producing residual fibrosis.

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Fig. 4. Adenine nucleotide depletion from cells prelabeled with ^{14}C -adenine, washed, and incubated with $20 \mu\text{M}$ H_2O_2 in serum-free medium for 10 or 30 min. Open bars indicate cellular adenine nucleotides; hatched bars indicate the sum of catabolic products (hypoxanthine, xanthine, uric acid) in the medium. Results are expressed as percentage of total soluble radioactivity in cells plus medium (means $\pm$ SD). Asterisks indicate $p < 0.05$ (Mann-Whitney U test, $n = 4$) compared with control (incubation for 30 min without H_2O_2).

Fig. 5. Adenine nucleotide depletion from cells prelabeled with ^{14}C -adenine, washed, and incubated with $100 \mu\text{M}$ H_2O_2 in serum-free medium for 10 or 30 min. Expression of results and symbols are as in Figure 4.

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