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NITRIC OXIDE MEDIATES TUMOR NECROSIS FACTOR-α CYTOTOXICITY IN ENDOTHELIAL CELLS

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SUMMARY: Tumor necrosis factor alpha (TNF-α) exerts multiple actions on endothelial cells including among others the expression of pro-coagulant activity and adhesion molecules, and secretion of cytokines. We now show that TNF-α induces a time- and dose-dependent cytotoxic effect on cultured bovine aortic endothelial cells. This TNF-induced cytotoxicity, which is preceded by increased production of nitric oxide (NO), is significantly decreased by the NO synthase inhibitor N-iminoethyl-L-ornithine (L-NIO). Dexamethasone, which prevents the expression of cytokine-induced NO synthase in endothelial cells, also inhibits TNF-α-dependent cytotoxicity. The results indicate that NO is involved in the cytotoxic effect of TNF-α on endothelial cells.

Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine secreted primarily by macrophages upon activation by bacterial endotoxin (1). TNF-α is a major mediator of endotoxin-induced shock (2,3) and other inflammatory reactions.

Endothelial cells exposed to TNF-α *in vitro* undergo several metabolic changes, including expression of procoagulant activity and surface adhesion molecules for leukocytes and the secretion of other cytokines, such as interleukin 1 (IL-1) and 6 (IL-6) (reviewed in 4). A toxic action of TNF-α on endothelial cells has been suggested (5), on the basis of the hemorrhagic necroses produced in certain tumors by *in vivo* administration of this cytokine (6), and the capillary leak syndrome related to endotoxemia (7). *In vitro*, a direct cytostatic effect of TNF-α on endothelial cell cultures has been reported (5,8), as well as an indirect toxicity mediated by polymorphonuclear leukocytes (9,10). However, the results as to whether TNF-α also has a direct cytolytic activity on these cells are inconclusive (5,11).

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Endothelial cells synthesize nitric oxide (NO) from the terminal guanidino nitrogen atom of L-arginine (12), in a reaction catalyzed by a Ca^{2+}- and calmodulin-dependent NO synthase (13), which is constitutively expressed in these cells. When stimulated by Ca^{2+} agonists, NO is transiently released from endothelial cells. Endothelium-derived NO activates guanylate cyclase in vascular smooth muscle cells and causes vascular relaxation (14), thus playing a major role in the control of local blood flow (15) and systemic blood pressure (16). Cytokine-activated macrophages also synthesize NO from L-arginine, by means of a different isoenzyme, which is not regulated by intracellular Ca^{2+} levels (17). Macrophage-derived NO is involved in tumor cell cytostasis and cytolysis by its interaction with iron-sulfur centers in a variety of enzymes related to energy production (18,19). In the presence of endotoxin (LPS) and interferon-γ (IFN-γ), endothelial cells also express a Ca^{2+}-independent NO synthase (20), with molecular and kinetic properties similar to those of the enzyme present in activated macrophages (17,21). This cytokine-induced endothelial NO shares the cytotoxic properties of the macrophage product, since endothelial cells activated *in vitro* with LPS and cytokines are able to damage themselves (22) or lyse cocultured metastatic tumor cells (23), and these effects are mediated by NO (22,23).

The purpose of this study is to analyze whether TNF-α has a direct cyotoxic action on endothelial cells, and to evaluate the participation of endothelium-derived NO in such an effect.

METHODS

Cell Cultures. Endothelial cells, obtained from bovine aortae by gentle scraping of the luminal surface, were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. Cells were characterized as endothelial by their cobblestone morphology, fluorescent acetylated low density lipoprotein uptake (24) and immunocytochemistry with the anti-endothelial antibody CLA 21/1 (Medac, Germany).

Cytotoxicity assay. Cells were seeded in 2 cm^2 tissue culture wells at a density of 50,000 cells/cm^2. Three days later, the cells were washed with PBS, and TNF-α was added in 1 ml serum free DMEM. Unless otherwise stated, cell-free supernatants were recovered 48 hours later, and lactate dehydrogenase (LDH) activity was measured by spectrophotometry, using NADH and sodium pyruvate as substrates. Cytotoxicity was calculated as the cell lesion index (CLI)

$$CLI = \frac{LDH_{TNF} - LDH_{control}}{LDH_{TX100} - LDH_{control}} \times 100$$

where LDH activity in the TNF-α-treated cell supernatant is expressed as the percentage of LDH in the medium of cells treated with 0.1% Triton-X100 (maximal release), after subtracting the LDH activity present in untreated cultures. When the effects of N-iminoethyl-L-ornithine (L-NIO) or dexamethasone were tested, these drugs were added to both control and TNF-α-treated cultures, at the same time as the TNF-α.

Nitrite production. The measurement of NO_2^- was based on the Griess reaction. 225 µl aliquots of cell-free supernatants were incubated with 75 µl of 2% sulfanilamide and 0.2% N-1-naphthylenediamine dihydrochloride in 1.2N HCl, in 96 well plates, at room temperature for 10 minutes, with shaking. NO_2^- concentration, proportional to OD_{540}, was determined using a microplate reader (EL 340, Bio-Tek) with reference to a standard curve.

Statistics. Data are expressed as mean ± SE. Student's t-test for unpaired samples was used for comparisons between groups. A value of $p < 0.05$ was considered significant.

Chemicals. Human recombinant TNF-α, dexamethasone, NADH and sodium pyruvate were from Sigma Chemical Co. (St. Louis, MO). L-NIO was a generous gift from Dr. Harold Hodson, Wellcome Research Laboratories, Beckenham, Kent, U.K.

RESULTS

Treatment with TNF-α (20 ng/ml) for 48 hours induced morphological alterations in endothelial cells (Figure 1, A and B). The cobblestone organization disappeared, some cells were retracted and showed elongated processes and others died and were floating in the medium. Exposure to TNF-α (2-100 ng/ml) also produced a significant and dose-dependent increase of the CLI in endothelial cell cultures with a maximal effect observed at 20 ng/ml (Figure 2 A). The increase in CLI was time-dependent, becoming significant after a 24 hour incubation period (Figure 2 B).

Bovine aortic endothelial cells spontaneously produced NO_2^-, which accumulated in the culture medium. In the presence of TNF-α, the NO_2^- concentration was significantly enhanced at 24 hours (Figure 3).

In order to investigate whether NO produced by endothelial cultures participates in the cytotoxicity of TNF-α in these cells, the effect of the specific NO synthase inhibitor L-NIO on control and TNF-α-treated cultures was studied. L-NIO reduced the CLI induced by TNF-α on endothelial cell cultures by 50% (Figure 4 A). In untreated cells, L-NIO produced a small but significant reduction in the LDH activity released to the culture medium (Figure 4B).

Dexamethasone, which has been shown to prevent NO synthase induction by TNF-α in a variety of cell types, including the endothelium (20), reduced the

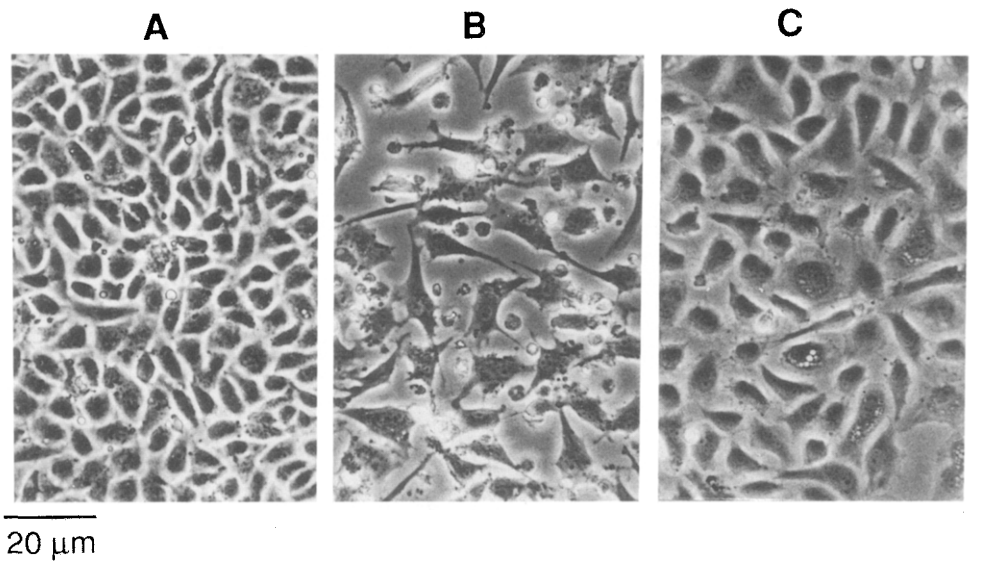

Figure 1. Phase photomicrographs of bovine aortic endothelial cells untreated (A) or treated with 20 ng/ml TNF-α (B), or TNF-α plus 1 μM dexamethasone (C), for 48 hours.

morphological alterations produced by TNF-α in endothelial cell cultures (Figure 1 C), and significantly inhibited its lytic effect (Figure 4 A). A reduction in basal LDH release in control cultures was also observed in the presence of dexamethasone (Figure 4 B).

DISCUSSION

The present results show that TNF-α has a direct cytolytic effect on cultured bovine aortic endothelial cells, as demonstrated by the increased activity of the

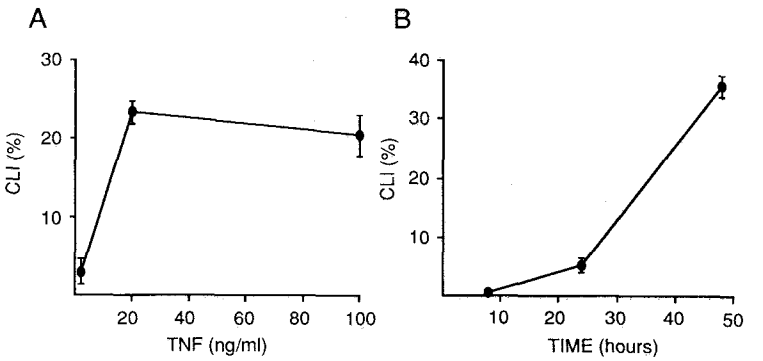

Figure 2. Cell lesion index (CLI) produced in bovine aortic endothelial cells incubated for 48 hours with different concentrations of TNF-α (A), or in cells incubated with 20 ng/ml TNF-α for different periods of time (B).

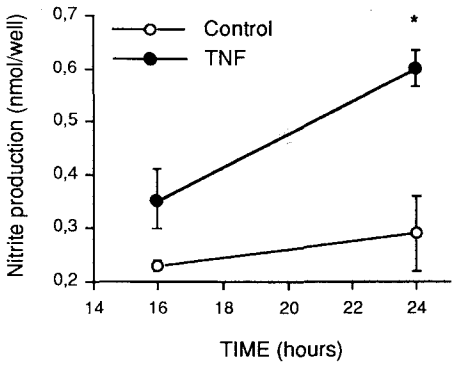

Figure 3. Nitrite production by bovine aortic endothelial cells in control conditions and in the presence of 20 ng/ml TNF-α.

cytoplasmic enzyme LDH in the culture medium. Although TNF-α has a cytostatic and cytolytic activity in a variety of tumor cell lines, non-transformed cells are generally resistant to the toxic effect of this cytokine (25). In endothelial cell cultures, TNF-α exerts a growth inhibitory action (5,8), which is mediated in part by a reduction of receptors for fibroblast growth factors (FGFs) (26). TNF-α also increases the susceptibility of endothelial cells to injury by activated polymorphonuclear leukocytes (9,10). However, results as to whether TNF-α is cytolytic for endothelial cells are

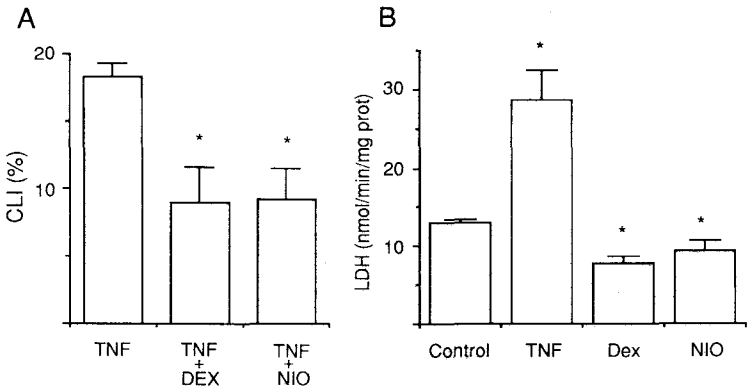

Figure 4. (A) Cell lesion index (CLI) of bovine aortic endothelial cells incubated for 48 hours in the presence of TNF-α (20 ng/ml), TNF-α plus dexamethasone (Dex) (1 μM), or TNF-α plus N-iminoethyl-L-ornithine (L-NIO) (100 μM). * $p < 0.05$, as compared with TNF-α treatment. (B) Lactate dehydrogenase activity measured in culture supernatants of control cells, and of cells treated for 48 hours with TNF-α (20 ng/ml), dexamethasone (Dex) (1 μM), or N-iminoethyl-L-ornithine (L-NIO) (100 μM). * $p < 0.05$, as compared with untreated cultures.
Experiments were performed in DMEM, containing 0.5 mM L-arginine. Data are the average of the results obtained in 6-8 wells. Experiments were repeated four times, with similar results.

controversial (5,11,27,28). Sato et al. (5) have reported a toxic action of TNF-α on bovine capillary endothelium, but not in cells derived from bovine aortae, whereas among pulmonary vessels, arterial but not microvascular endothelial monolayers were shown to be sensitive to TNF-α toxicity (11). A possible reason why TNF-α toxicity on endothelial cells was not observed in some of these studies is that analyses of the lytic effect were performed up to 24 hours after addition of the cytokine. According to the present data, when 24 hour incubations were used, the number of lysed cells was below 10%, and such a small effect can only be detected when the method used to evaluate cell death is sensitive enough (28). When exposure to TNF-α was extended for up to 48 hours, the cell damage increased significantly and could be clearly appreciated both by LDH measurement and by microscopic observation.

Lipopolysaccharides and cytokines, in combination with IFN-γ, have been shown to produce a sustained release of NO in cultured endothelial cells (20, 29). This effect is mediated by the induction in endothelial cells of a Ca^{2+}-independent NO synthase (20) similar to the enzyme present in activated macrophages (17,21). The present experiments show that TNF-α alone can also induce NO synthesis in bovine aortic endothelium, the release being maximal between 16 and 24 hours. This is in agreement with a recent report showing that TNF-α enhances the formation of L-[^{14}C] citrulline from L-[^{14}C] arginine in bovine endothelial cells in culture, and increases the concentration of cGMP in cocultured mesangial cells (30).

Cytokine-induced endothelial NO has been shown to produce cell lysis in cocultured tumor cells (23), an effect similar to that of the NO released by activated macrophages (19). An autocytotoxic effect of NO generated by the inducible NO synthase has recently been reported in endothelial cells stimulated with LPS and IFN-γ (22). Endothelium-derived NO also mediates the toxic effect of TNF-α on cultured endothelial cells, as is clearly demonstrated by the following observations: a) L-NIO, a potent and irreversible inhibitor of the macrophage type NO synthase (17), significantly inhibited TNF-α induced toxicity, and b) dexamethasone, which has been shown to prevent expression of inducible NO synthase in endothelium (20), also reduced the damage of endothelial cells by TNF-α, both by morphologic criteria and when LDH activity was evaluated.

The decrease in LDH activity measured in culture media when cells were incubated, in the absence of TNF-α, with L-NIO or with dexamethasone, suggests that, in standard culture conditions, there may be some induction of NO synthase. This fact could be due to the presence of small amounts of endotoxin in the culture media, and

may explain some of the spontaneous NO_2^- production observed in the absence of TNF-α.

The production of NO by TNF-α-treated endothelial cells, and the toxicity of this NO towards both tumoral and endothelial cell lines, may explain, at least in part, the tumoricidal effect of TNF-α when administered *in vivo* (6). The NO-mediated toxic effect of TNF-α on endothelial cells may also be relevant in those situations in which TNF-α is elevated in plasma, such as endotoxin-induced shock. In this condition, endothelial lesions would allow blood components to contact the basal lamina, thus favoring thrombus formation. In addition, circulating TNF-α could act on the underlying smooth muscle cell receptors, inducing NO synthesis also in muscle cells, and thus contributing to the vascular relaxation and the severe hypotension that occurs in endotoxemia.

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