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PRODUCTION OF AN ARGININE-DERIVED RELAXING FACTOR INDUCED BY IFN-γ PLUS ENDOTOXIN IN MURINE ADENOCARCINOMA EMT 6 CELLS

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SUMMARY : Treatment of EMT 6 mammary adenocarcinoma cells with Interferon-γ (IFN-γ, 10 $U.ml^{-1}$) plus endotoxin lipopolysaccharide (LPS, 100 $ng.ml^{-1}$) induces concomitantly a growth arrest and production of citrulline and nitrite from L-arginine. A similar L-arginine-dependent metabolism is responsible for the vascular smooth muscle relaxing effect of stimulated endothelial cells. We therefore investigated the ability of EMT 6 cells to induce the relaxation of endothelium-denuded rat aortic rings precontracted with noradrenaline (1 μM). Pretreatment of EMT 6 cells with IFN-γ+ LPS increased their relaxing potency by 5-10 times. The relaxing effects of control and treated EMT 6 cells were entirely counteracted by N^G-monomethyl-L-arginine (300 μM), a specific inhibitor of nitrite and citrulline production from L-arginine, and by methylene blue (10 μM) and LY 83583 (10 μM), two inhibitors of NO°-induced activation of guanylate cyclase. The effect of N^G-monomethyl-L-arginine was reversed by L- but not D-arginine (1 mM). It is concluded that IFN-γ + LPS increase the production of a relaxing factor in EMT 6 cells through the L-arginine-NO°-synthase pathway.

A metabolic pathway leading from arginine to NO° and citrulline has been unraveled recently in cells of the vascular endothelium (1, 2) and activated macrophages (3, 4, 5, 6) or macrophage cell lines (7, 8).

In endothelial cells, this pathway is constitutive and is activated upon reception of adequate signals such as acetylcholine or bradykinin (1). Its main effector molecule, NO°, induces relaxation of the smooth muscle cells of the vessel through the activation of cytosolic guanylate cyclase and further accumulation of guanosine 3', 5' monophosphate (cyclic GMP) (2) : it is called EDRF (Endothelium-Derived Relaxing Factor). In macrophages and related cell lines, this pathway has to be induced by activation signals such as Interferon-γ (IFN-γ) plus endotoxin lipopolysaccharide (LPS) and remains in function permanently until the macrophage becomes deactivated; it generates a molecule with EDRF like activity, probably NO° (4, 5) which seems also to be the effector molecule of the cytostatic activity against tumor cells (8).

A growth arrest can be induced by IFN-γ and LPS (9, 10), or a monokine (9) in cells of the EMT 6 murine mammary adenocarcinoma. We have previously reported that this cytostasis is L-arginine-dependent and is associated with an increased accumulation of citrulline and of nitrite, a stable metabolite of NO°, in the culture medium (10).

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To further investigate this pathway in a non-macrophage non-endothelial cell line, we looked for the production of an EDRF-like molecule by untreated EMT 6 cells and by EMT 6 cells treated with IFN-γ and LPS. EMT 6 is an adherent cell line, therefore we used cells grown on carrier beads detached by pipetting. We introduced them *directly* into the organ chamber containing a contracted endothelium-denuded rat aortic ring.

MATERIAL AND METHODS

Preparation of EMT 6 cells : EMT 6 is a murine mammary adenocarcinoma from the BALB/c mouse provided by Dr Lopez-Berestein (Anderson Hospital and Tumour Institute, Houston, TX). EMT 6 cells were grown in RPMI 1640 medium (Gibco, Cergy-Pontoise, France) with L-glutamine and 25mM Hepes, supplemented with antibiotics and 5 % foetal calf serum containing less than 0.11 $ng.ml^{-1}$ LPS (Gibco). They were detached by trypsin plus EDTA before each biweekly passage. For the experiments reported in this paper they were grown on Biosilon (Nunc, Denmark) microcarrier beads in standard, bacteriology grade Petri dishes. Under these conditions the cells adhered only to the carrier beads, from where they could be detached by repeated pipetting. The cell suspension obtained was washed twice with Krebs-bicarbonate solution. Growth arrest of EMT 6 cells was achieved by addition of 10 $U.ml^{-1}$ recombinant murine IFN-γ (from Genentech, kindly provided by Dr G.R. Adolf, Ernst Boehringer Institut, Wien, Austria) and 100 $ng.ml^{-1}$ LPS (from *Salmonella enteritidis*, Difco, Detroit, MI) as described previously (10). Nitrite concentration in EMT 6 culture medium was measured with the Griess reagent (10).

Relaxation of endothelium denuded rat aortic rings : Male Wistar rats (10-12 weeks old) were killed by cervical dislocation. Thoracic aortae were dissected and cut into rings of 2-3 mm. The endothelium was removed by gently rubbing the intimal surface with a small wooden stick. The rings were mounted under 2g tension in organ baths containing 10 ml Krebs-bicarbonate solution (NaCl 118 mM ; NaHCO3 25 mM ; glucose 10 mM ; KCl 4,7 mM ; CaCl2 1,25 mM ; MgSO4 1,19 mM ; KH2PO4 1,14mM) aerated with 95 % O2 - 5%CO2 and maintained at 37°C.
After 60 min equilibration with regular washing, noradrenaline (1 μM) (Sigma) was added to each bath. When the contraction was fully developed, the absence of functional endothelium was tested by the absence of any relaxation upon the addition of acetylcholine (1 μM).(Sigma).
After a further washing period of 60 minutes the tissues were incubated for 15 min with superoxide dismutase (SOD) (100 $U.ml^{-1}$) (Sigma) prior to readdition of noradrenaline (1 μM). SOD was present during subsequent experiments in order to protect NO° from degradation by superoxide anions.When the contraction was stable, treated or control EMT 6 cells were cumulatively added to the baths, each addition being made when the relaxation had reached an equilibrium. After maximal relaxation was reached N^G-monomethyl-L-arginine (L-NMMA) (300 μM) (Calbiochem), D-arginine (Sigma) (1 mM) and L-arginine (1 mM) (Calbiochem) were successively added to explore the arginine dependence of the relaxation.
In experiments designed to investigate the involvement of NO°-induced activation of guanylate cyclase, methylene blue (10 μM) (Sigma) an inhibitor of guanylate cyclase or LY 83583 (6-anilino-5,8-quinolinedione) (Eli Lilly & Co ; Indianapolis, USA) which decreases NO° release and is also a direct inhibitor of guanylate cyclase at the used concentration (10 μM) (11, 12) were added to aortic rings relaxed with EMT 6 cells.
Aortae from four different rats were used in each experimental condition and the results are expressed as the mean ± standard deviation of the mean. Statistical comparisons between contraction of the same aortic rings before and after the addition of EMT 6 cells were made using the paired t-test . A P value ≤ 0.05 was considered to be significant.

RESULTS

Representative traces illustrating the experimental protocol and the relaxing effect of IFN-γ plus LPS-treated EMT 6 cells are shown in figure 1.

Both control and activated EMT 6 cells produced relaxation of norepinephrine-contracted aortic rings in a cell concentration-dependent fashion (figure 2). However, at a concentration of $0.4x10^5$

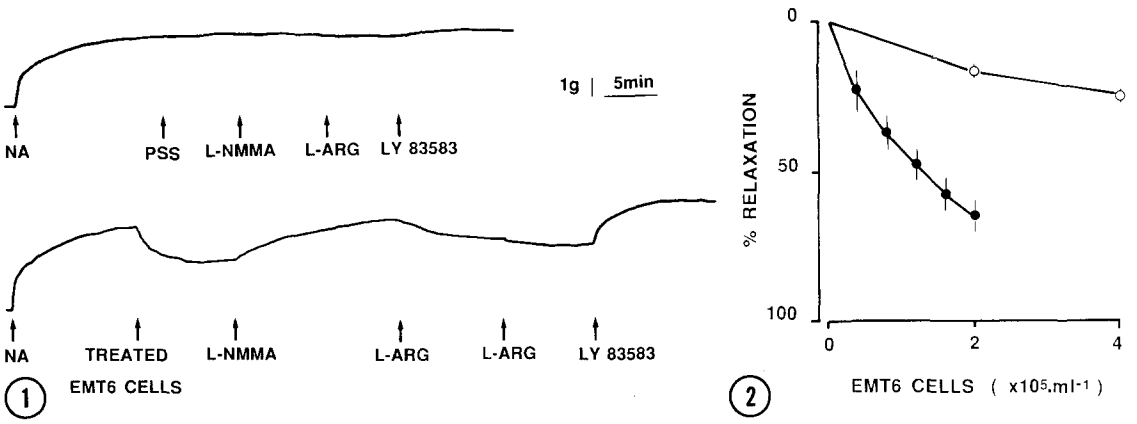

Fig. 1 . Representative traces illustrating the NO°-like relaxing effect of EMT 6 cells treated with IFN-γ plus LPS.
Activated EMT 6 cells (2,5 x 10^5 x ml^{-1} final concentration) or the equivalent volume of Krebs physiological salt solution (PSS, 100 µl) were added to endothelium-denuded rat aortic rings precontracted with noradrenaline (1 µM). The following reagents were then successively added, in final concentrations : L-NMMA (300 µM) L-arginine (1mM and 3mM), LY 83583 (10 µM).

Fig. 2 . Relaxing effect of EMT 6 cells on endothelium-denuded rat aortic ring.
Control (o) or IFN-γ plus LPS-treated (•) EMT 6 cells were cumulatively added to aortic rings precontracted with noradrenaline (1 µM). Relaxation was expressed as% of noradrenaline induced contraction (2,9 ± 0,3 g and 3,4 ± 0,3 g in aortic rings respectively used with control and activation cells). The abscissa represents the final concentration of cells added to the organ bath.

cells x ml^{-1}, activated cells produced a relaxation (23 %) which was not statistically different from relaxations produced by 2 and 4x10^5 control cells x ml^{-1} (17 and 25 %, respectively). Thus, the pretreatment of EMT 6 cells by IFN-γ plus LPS increased their relaxing potency by 5-10 times.

As illustrated in figures 1 and 3, relaxation induced by control or activated EMT 6 cells was markedlly reduced by L-NMMA. Subsequent addition of D-arginine had no effect, but L-arginine restored the relaxing effect of the cells, demonstrating a stereospecific L-arginine requirement. Furthermore, relaxation produced by EMT 6 cells was abolished by two inhibitors of cyclic GMP production, methylene blue (figure 3) and LY 83583 (figure 1) : after the addition of one or the other compound, contraction of aortic rings returned to the levels initially produced by noradrenaline alone, showing that EMT 6 cells-induced relaxation was mediated by cyclic GMP, which is also responsible for the effect of EDRF.

In order to monitor the background and IFN-γ plus LPS activated production of NO° by EMT 6 cells, the accumulation of nitrite, a stable derivative of NO°, was investigated in the culture medium. The nitrite concentration found in the conditioned medium from control EMT 6 cells grown on beads was 11 µM, compared with 40 µM for the cells grown in the same conditions but activated with IFN-γ plus LPS during the last 18h of culture. This 4 times increased nitrite accumulation during the whole pre-incubation time is in good agreement with the 5-10 times increased relaxing potency at the end of the activating pre-treatment with IFN-γ plus LPS. In contrast with control cells grown on beads, no nitrite was detected in the culture medium of untreated EMT 6 cells grown under standard conditions of adherence to plastic dishes (10). The same phenomenon of partial activation of NO° production upon growth on carrier beads has been reported for the RAW macrophage cell line (4).

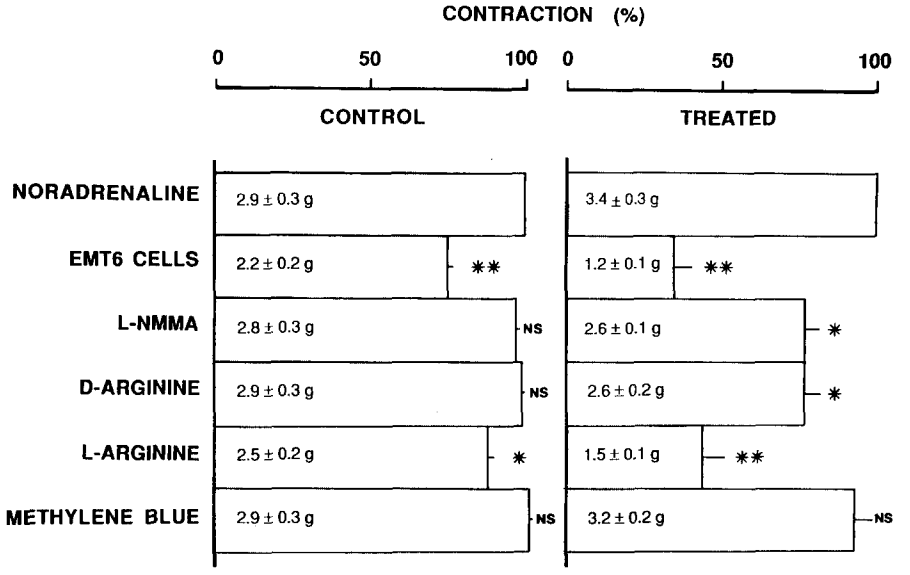

Fig. 3.Implication of the L-arginine-NO° pathway in the relaxing effect of EMT 6 cells.
Control (4 x 10^5 x ml^{-1}) or IFN-γ plus LPS-treated (2 x 10^5 x ml^{-1}) cells were added to noradrenaline precontracted aortic rings (for experimental conditions, see figure 2). The following reagents were then successively added : L-NMMA (300 μM) D-arginine (1 mM), L-arginine (1 mM) methylene blue (10 μM) (final concentration). Each reagent was added when the previous one had reached its maximal effect, using the experimental protocol illustrated in figure 1. Contraction is expressed as % contraction induced by noradrenaline (1 μM).
The data between parenthesis contained in each column indicates absolute values of contraction (in grams) in the corresponding experimental condition. * ($P \leq 0.05$) ** ($P \leq 0.01$) indicate statistically significant differences with noradrenaline induced contraction before the addition of EMT 6 cells (100 %) (N.S., not statistically significant).

DISCUSSION AND CONCLUSION

Activation of macrophages by interleukins and LPS is well documented. We previously reported that IFN-γ plus LPS also activate the non macrophage EMT 6 cell line, inducing a growth arrest and a concommitant accumulation of nitrite and citrulline in the culture medium (10). A metabolic pathway producing the free radical NO° and citrulline from L-arginine has been characterized in macrophages (3, 1), in endothelial cells (1, 2) and in other cells (5, 9, 13, 14, 15). NO° is rapidly oxidized into nitrite and nitrate (2, 8), but it possesses a potent biological activity which may account for EDRF activity in blood vessels and probably for the cytostatic properties of macrophages (8). We therefore asked the question whether IFN-γ plus LPS treatment of EMT 6 cells activates the L-arginine-NO° pathway, leading to production of an EDRF-like factor. The results reported here show that this is the case, since EMT 6 cell smooth muscle relaxing activity is associated with the accumulation of nitrite in the culture medium, is increased by the treatment with IFN-γ plus LPS, is blocked by the NO°-synthase inhibitor L-NMMA in an L-arginine reversible manner and is abolished, like NO° relaxing activity by methylene blue and LY 83583. The latter compound has been shown to inhibit soluble guanylate cyclase at relatively high concentrations ($\geq$ 0.3 μM) and also to decrease the release of EDRF, at lower concentrations, by generating superoxide ions (11, 12).In experiments not reported here, 1 μM LY 83583 decreased by 27 % the production of nitrite by stimulated EMT 6 cells cultured for 24 h.

The existence of cytosolic enzyme activity responsible for NO° production from L-arginine (L-arginine-NO°-synthase) has been demonstrated in endothelial cells (16, 17) and macrophages (18). It is strictly dependent on NADPH and its activity, at least in macrophages, requires the presence of constitutive and non constitutive components (18). In addition, there seems to be tissue differences, for instance in the sensitivity to divalent cations (19). In view of the results reported here, we think that the EMT 6 cell line is a particularly good model to study the L-arginine-NO° synthase pathway and the mechanisms of its effector molecules.

In conclusion, the present work shows that the adenocarcinoma EMT 6 cells are able to produce a vascular relaxing factor through the L-arginine-NO° synthase pathway and that a cytostatic treatment of these cells with IFN-γ plus LPS is associated with an increased production of this factor. These findings may have important pathological implications with respect to the irrigation of the tumor and the antitumoral effects of signals activating this pathway.

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