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SPECIAL REPORT

Involvement of nitric oxide in the inhibition of angiogenesis by interleukin-2

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Interleukin-2 (IL-2), an immunoregulatory cytokine possessing antitumour activity, is an inducer of nitric oxide (NO) synthesis in mice and man. In this study, the possibility that IL-2 possesses antiangiogenic properties that account for its antitumour effects *in vivo* was examined. IL-2 caused a dose-dependent inhibition of angiogenesis in the chick embryo chorioallantoic membrane (CAM). This inhibition was completely reversed by the NO synthase inhibitor N^G-nitro-L-arginine methylester (L-NAME). Furthermore, IL-2 was capable of stimulating NO synthase activity in the CAM *in vitro* and this effect was suppressed by L-NAME. Addition of IL-2 to human umbilical vein endothelial cells (HUVECs) in culture, had no effect on their growth characteristics. These results suggest that IL-2 may be an important antiangiogenic molecule causing its effect via nitric oxide synthesis. The antiangiogenic activity of IL-2 may be, at least in part, responsible for its antitumour properties.

Keywords: Interleukin-2 (IL-2); angiogenesis; nitric oxide (NO); nitric oxide synthase (NOS); chick chorioallantoic membrane (CAM)

Introduction Interleukin-2 (IL-2) is an immunoregulatory molecule, which by interacting with specific receptors induces differentiation and proliferation of T-lymphocytes (Whittington & Faulds, 1993). This process stimulates a cascade responsible for its antitumour activity. IL-2 is a potent inducer of nitric oxide (NO) synthase, at the level of transcription (Deng *et al*., 1993). Subsequent studies have shown that NO synthesis may contribute to the IL-2 induced antitumour responses (Yim *et al*., 1995; Hibbs *et al*., 1992). We have previously shown that NO possesses antiangiogenic and antitumour properties (Pipili-Synetos *et al*., 1995). Since IL-2 activity may be partly mediated via NO, the question arose as to whether the effects of IL-2 on tumour growth may be related to inhibition of angiogenesis. The effect of IL-2 alone and in combination with the NO synthase (NOS) inhibitor N^G-nitro-L-arginine methylester (L-NAME) was therefore examined. It was found in the chick embryo chorioallantoic membrane (CAM), an *in vivo* model of angiogenesis, that IL-2 inhibited angiogenesis and this effect appeared to be mediated via NO synthesis.

Methods The *in vivo* CAM angiogenesis model, initially described by Folkman (1985) and modified by Maragoudakis *et al*. (1988), was used. Biochemical evaluation of angiogenesis was performed by determining the extent of collagenous protein biosynthesis in the CAM lying directly under the disc. The tissue containing radioactivity was subjected to collagenase digestion. The resulting radiolabelled tripeptides corresponding to basement membrane collagen and other collagenous material synthesized by the CAM from [U-^{14}C]-proline, were counted and expressed as c.p.m. mg^{-1} protein. For each egg, collagenous protein biosynthesis under the disc containing the test material was then expressed as % of that under the control disc in the same egg (*n* signifies the number of eggs for each treatment).

For morphological evaluation, eggs were treated as above in the absence of radiolabelled proline. At day 11, eggs were flooded with 10% buffered formalin. An area around the discs was cut off, placed on a glass slide and the vascular index was measured by use of a computer-assisted Image Analysis System (Maragoudakis *et al*., 1995).

For the proliferation assay, human umbilical vein endothelial cells (HUVECs) (passage 3) were synchronized by serum starvation for 24 h in medium 199 (M199) containing 0.2% bovine serum albumin. Subsequently, the cells were removed and seeded at 5×10^4 cells/well in 12 well tissue culture plates in M199 containing 10% foetal calf serum (FCS). The test substances were added and the cells were cultured at 37°C for 48 h. Cells were dispersed with 0.25% trypsin and counted in a haematocymeter.

NOS activity was measured as citrulline formed from [^{14}C]-arginine in the CAM *in vitro* according to the method of Chigo *et al*. (1995). Briefly, at day 10, CAMs from 20 eggs were incubated for 24 h with the test materials or the vehicle. The tissues were then homogenized in an appropriate buffer and centrifuged at 10 000 *g* for 30 min at 4°C. Each sample (400 ìl) of the supernatants was added to triplicate tubes containing 0.5 μCi L-[^{14}C]-arginine, 1 mM NADPH, 0.8 mM $CaCl_2$, 15 μM L-arginine, 25 mM L-valine, and when indicated, 0.1 M L-NAME or 1 mM EGTA. After 10 min incubation at 37°C, the reaction was stopped with 3 ml HEPES-Na^+, pH = 6, containing 2 mM EDTA. The reaction mixture was passed through a 1 ml Dowex-Na^+ column and activity was eluted with 1 ml of distilled H_2O. L-[^{14}C]-citrulline related radioactivity in 1 ml of eluate was measured by liquid scintillation counting. Specific NOS activity was expressed as pmol citrulline min^{-1} mg^{-1} protein (*n* signifies the number of experiments).

Materials Fertilized eggs were obtained locally (Ioannina, Greece). Tissue culture media were obtained from SEROMED; L-[^{14}C]-guanido-arginine was from Dupont. L-[U-^{14}C]-proline was from New England Nuclear (Boston, MA). All the other reagents were obtained from Sigma Chemical Co. (Poole). Interleukin-2 was a kind gift from Dr E. Baxevanis (Agios Savas Anticancer Institute, Athens, Greece).

Results Interleukin-2 (IL-2) from 100–1000 iu/disc caused a dose-dependent inhibition of basal angiogenesis as shown by a decrease in collagenous protein biosynthesis in the CAM, ranging between 13.7 ± 4% and 33.5 ± 3.4% of control ($n = 15–17$) (Figure 1a). Higher amounts of IL-2 (3000 iu/disc) did not result in further inhibition of angiogenesis

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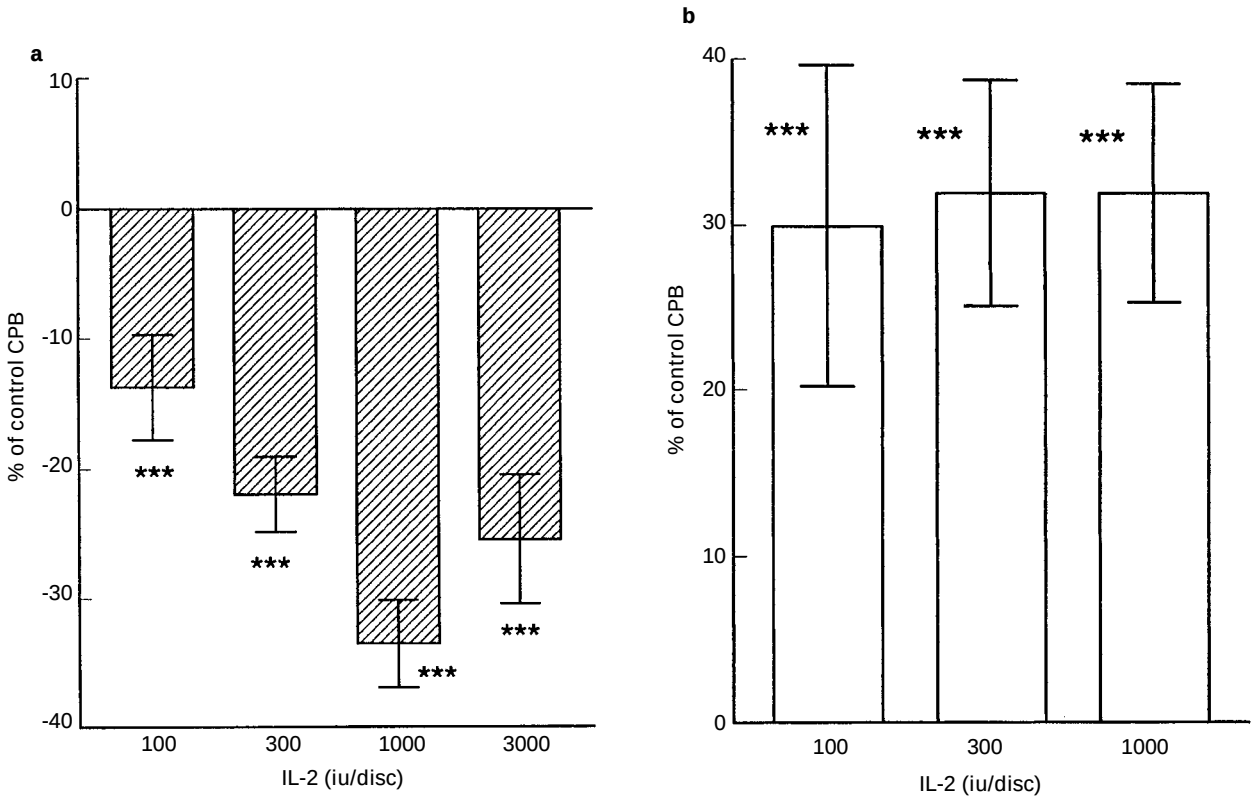

Figure 1 Effect of increasing amounts of interleukin-2 (IL-2) on angiogenesis in the chick chorioallantoic membrane (CAM) *in vivo* expressed as collagenous protein biosynthesis (CPB) in the absence (a) and presence (b) of 34.2 nmol/disc of the NO synthase inhibitor N^G-nitro-L-arginine methylester (L-NAME). Results are expressed as mean $\pm$ s.e. of the mean % of control and were compared by paired *t* test (***$P<0.01$). The number of observations is indicated in the text.

($25.4\pm5\%$ of control, $n=9$); this was probably due to an inflammatory reaction under the disc containing the test material giving false positive results. Morphological evaluation showed that the vascular density under the discs containing 1000 iu was reduced by $18.1\pm5.4\%$ of control, $n=14$, $P<0.01$.

IL-2 at concentrations ranging from 30–1000 iu ml^{-1} had no significant effect on the proliferation of HUVECs ($33.0\pm1.6\times10^3$ cells/well, $n=6$, in the absence, compared to $28.0\pm1.5\times10^3$ cells/well, $n=6$, in the presence of IL-2).

In agreement with previous observations (Pipili-Synetos *et al*., 1994), L-NAME (34.2 nmol/disc) stimulated angiogenesis, as evidenced by an increase in collagenous protein biosynthesis by $31.9\pm6.6\%$ of control ($n=6$). When IL-2 (100–1000 iu/disc) was combined with L-NAME, the antiangiogenic effect of the former was completely abolished and an increase in collagenous protein biosynthesis was observed ranging between $28.5\pm8.3\%$–$31.9\pm6.8\%$ of control ($n=12$–15) (Figure 1b). The extent of this increase was comparable to that seen with L-NAME alone. Morphological evaluation showed that the vascular density under the discs containing IL-2 and L-NAME was increased by $27.3\pm3.1\%$ of control, $n=6$, $P<0.01$.

The ability of IL-2 to induce synthesis of NO in this tissue was then examined. When CAMs from 20 eggs were preincubated with IL-2 from 100–1000 iu ml^{-1}, citrulline synthesis was shown to increase progressively and at 1000 iu ml^{-1}, it reached 14.8 ± 0.3 pmol min^{-1} mg^{-1} compared to 6.5 ± 0.7 pmol min^{-1} mg^{-1} in the control CAM ($n=2$). EGTA addition resulted to the same amount of citrulline synthesis (14.4 ± 0.6 pmol min^{-1} mg^{-1} in support of the induction of NO synthesis by IL-2.

Discussion In the present study it was shown that in the *in vivo* CAM model, IL-2, a cytokine with antitumour effects (Whittington & Faulds, 1993), inhibited angiogenesis. This effect was completely abolished by L-NAME, the NOS inhibitor, suggesting that the antiangiogenic effect of IL-2 was mediated through NO synthesis. Furthermore and in agreement with these observations, IL-2 stimulated the activity of the inducible NO synthase in the CAM *in vitro*.

In view of the fact that tumour growth depends on new vessel formation, the antitumour effect of IL-2 may be a consequence of its antiangiogenic properties. This study therefore provides evidence that inhibition of angiogenesis may be included in the long list of biological properties exerted by IL-2. Interleukin-2 receptors have been identified on HUVECs (Hicks *et al*., 1991) and their numbers on the cell surface vary according to passage and culture conditions. Interleukin-2 had no effect on the growth rate of HUVECs under the conditions used here. Whether this is related to iNOS expression following IL-2 receptor activation in these cells, cannot be concluded from the present experiments. However, our results provide corroborative evidence that the inhibitory effect of IL-2 on angiogenesis may not be due to direct inhibition of endothelial cell proliferation.

Nitric oxide formation may be an important regulator of the angiogenic process under basal conditions in the CAM, since L-NAME can stimulate this process (Pipili-Synetos *et al*., 1994). In addition NO donors exert antitumour and antimetastatic effects in mice bearing Lewis Lung carcinoma (Pipili-Synetos *et al*., 1995). Since NO appears to mediate the antiangiogenic effect of IL-2, the importance of NO as a modulator of angiogenesis is further supported and as such it may be a target for the control of angiogenesis-dependent disease.

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