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INVOLVEMENT OF NITRIC OXIDE ON THE CYTOKINE INDUCED GROWTH OF GLIAL CELL

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SUMMARY: The growth of C6 glioma cells was stimulated by TNF-α, IFN-γ and IL-6 but not by TNF-β. However, TNF-α and IFN-γ but not IL-6 induced the synthesis of NO in C6 cells. Moreover, N-monomethyl-L-arginine a competitive inhibitor of NO synthase blocked TNF-α and IFN-γ-dependent proliferation and NO induction on C6 cells, but had no effect on IL-6-dependent proliferation. In addition, C6 proliferation induced by TNF-α and IFN-γ was specifically blocked by inhibitors of cyclic nucleotide dependent protein kinases such us H-9. Those results suggest that TNF-α and IFN-γ but not IL-6 induce the growth of glia cells through the generation of NO which in turns activate a cyclic nucleotide dependent kinase.

Cytokines are highly pleiotropic soluble molecules which play a very important role controlling many body functions including the nervous system (1). Cells from CNS, such as astrocytes and microglial cells, have been shown to secrete a variety of cytokines including IL-6 (2) and TNF-α (3). In addition, cytokines have important regulatory effects on the growth and differentiation of cells from the CNS. Thus, IFN-γ induces MHC class II expression on astrocytes (4) and IL-6 induces differentiation of PC-12 cells (5). Moreover,TNF-α has a broad range of effects on the CNS, including inducing MHC class I and II expression (6), IL-6 secretion (7), and proliferation (8) in astrocytes.

On the other hand, NO has been characterized as the endothelium relaxing factor which is synthesized from L-arginine by the NO synthase. Two types of NO synthase have been clearly characterized. One is constitutive, Ca2+/calmodulin-dependent present in neurones and in endothelial cells and the other is Ca2+/calmodulin-independent found in macrophages and another cell types (reviewed in 9,10). NO synthesis in macrophages is induced after

Abbreviations: CNS, Central nervous system; FCS, fetal calf serum; D- or L-NMMA, N^G-monomethyl-D- or L- arginine; PK, protein kinase; TNF, Tumor necrosis factor.

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treatment with TNF-α and IFN-γ (11,12). NO may act as also a neuronal messenger (9,13). Stimulation of glutamate receptors increases NO synthesis which in turn promotes cGMP accumulation through activation of guanylate cyclase in the cerebellum (13). We have previously shown that TNF-α modulated the growth of several cells of the nervous system including C6 glial cells (14). We have compared here the effects of some cytokines on C6 growth and characterized their molecular mechanism of action.

MATERIALS AND METHODS

Reagents. Recombinant human IFN-γ and IL-6 were a generous gift of Dr. Luis Carrasco, Universidad Autonoma de Madrid, Spain and Dr. Walter Fiers, University of Ghent, Belgium, respectively. Recombinant human TNF-α and TNF-β were supplied by Genentech, Inc. (South San Francisco, CA). L- and D-NMMA were from Calbiochem-Behring Corp., (La Jolla, CA). H-7 and H-9 were from Research Biochemichals Inc. (Natick, MA)

Determination of cell growth. Rat glioma C6 cells (10^5 cells/ml in DMEM containing 10% FCS), were cultured in 24-well microtiter plates and treated with different concentrations of recombinant cytokines for the indicated times. Cell number was evaluated by counting viable cells. DNA synthesis was estimated by measuring [^{3}H]thymidine (New England Nuclear, Boston, MA) incorporation in triplicate cultures of 10^4 C6 cells in 0.1 ml of DMEM containing 10% FCS, incubated for 24 h in the presence of cytokines, as decribed (14).

NO production. NO formation was detected by NO_2^- accumulation in culture supernatants using the Griess reaction (15). Briefly, 50 µl aliquots of medium were incubated with 50 µl of 1% sulfanilamide and 50 µl of 1% N-1-naphthylethylenediamide dihydrochloride (Sigma Chemical Co., St Louis, Mo) in 2.5% H_3PO_4 at room temperature for 5 min. Optical density at 543 nm was measured and NO_2^- was quantified by comparison to $Na(NO_2)$ as standard.

RESULTS AND DISCUSSION

Transformed cell lines represent a good model to study cell differentiation in the nervous system. C6 glial cells were cultured in presence of different doses of cytokines and the viable cell number was determined after 24, 48 or 72 h of culture. TNF-α, IL-6 and IFN-γ were able to significantly increase C6 growth rate even in saturating serum conditions at all time points. By contrast, TNF-β did not affect C6 growth (Figure 1A). The effect of TNF-α and IL-6 was blocked by neutralizing monoclonal anti-human TNF-α and anti-IL-6 antibodies respectively (data not shown). Furthermore, there seem to be an additive effect of IFN-γ with low doses of TNF-α in the enhancement of C6 growth.

The above results were confirmed measuring [^{3}H]thymidine incorporation into DNA. TNF and IFN-γ were able to significantly increase C6 DNA synthesis at low doses (10 U/ml) being the optimal dose 100 U/ml. By contrast, IL-6 was less potent than IFN-γ and TNF-α requiring doses up to 1000 U/ml to reach maximum stimulation. Again TNF-β had no effect even as doses as high as 1000 U/ml (Figure 1B).

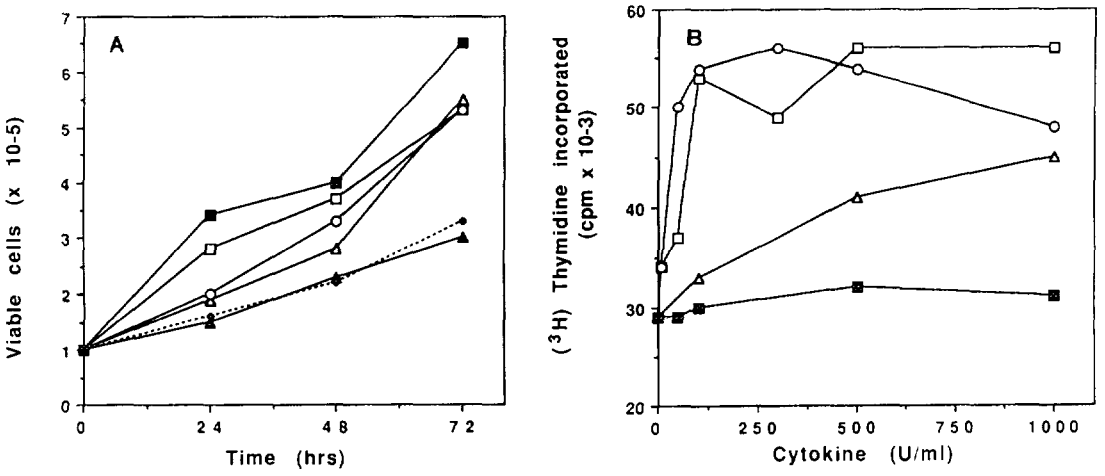


Figure 1. Induction of C6 cell growth by cytokines. (**A**) C6 cells were cultured in absence of any cytokine (◊) or in presence of TNF-α (200 U/ml) (□), IFN-γ (50 U/ml) (○), IL-6 (500 U/ml (△), TNF-β (1000 U/ml) (▲) or a combination of TNF-α plus IFN-γ (■). The number of viable cells was evaluated at the indicated times. (**B**) Induction of DNA synthesis in C6 cells by cytokines. C6 cells were cultured for 24 hrs in presence of the indicated concentrations of TNF-α (□), IFN-γ (○), IL-6 (△) or TNF-β (■). DNA synthesis was estimated by measuring [^{3}H] thymidine incorporation during the last 4 h of cultures.

TNF-α and IFN-γ have been recently shown to synergize in macrophage activation through the induction of NO synthesis (11,12). In order to study if similar mechanism was operating in glial cells, we tested the effect of cytokines on NO synthesis in those cells. As shown in Table 1, TNF-α and IFN-γ but not IL-6 or TNF-β induced NO formation in C6 cells. The induction by TNF-α and IFN-γ was greater than by any cytokine alone. This effect was completely blocked by L-NMMA, which is a known competitive inhibitor of the NO

TABLE I. NO formation by C6 cells

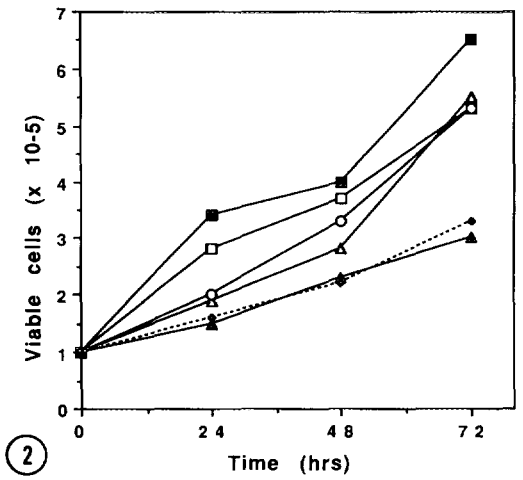
Stimulus	NO^{2-} produced (μmol/10^6 cells)		
	Control	+L-NMMA	+ D-NMMA
None	6+/-3	11+/-4	12+/-4
TNF-α (500 U/ml)	72+/-8	10+/-3	85+/-9
IFN-γ (100 U/ml)	90+/-8	15+/-4	90+/-9
IL-6 (500 U/ml)	12+/-4	9+/-3	14+/-4
TNF-α (500 U/ml) + IFN-γ (100 U/ml)	116+/-9	19+/-4	120+/-8

C6 glia cells were stimulated with cytokines in the absence of (control) or in presence of 100 μM L- or D-NMMA. The NO produced was determined 72 h later. Results shown are the means +/- S.E. of two different experiments.

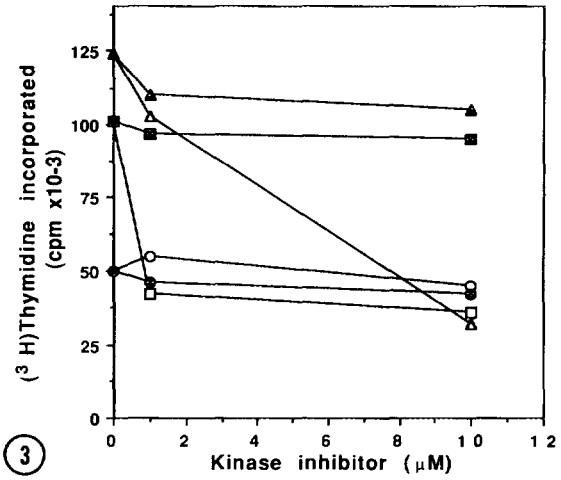
synthase (9,10) but not by its inactive enantiomer D-MMLA. To test if the NO produced was involved in the activity of the cytokines or was a mere byproduct, the effect of L-NMMA on cytokine-induced proliferation was tested. L-NMMA but not D-NMMA blocked in a dose response manner the growth induced by TNF-α and IFN-γ or by a combination of both cytokines. As expected, L-NMMA had no effect on IL-6 activity or the basal growth rate (Figure 2).

Since NO has been shown to activate cGMP formation (13) which in turns activate cGMP-dependent PK, we tested the effect of PK inhibitors on cytokine activity. H-9 a preferential inhibitor of cyclic nucleotide-dependent PK inhibited in a dose response manner both the IFN-γ and the TNF-α-induced DNA synthesis of C6 cells. By contrast, H-7 which shows preference for PK-C had no effect. Furthermore, H-9 had no effect on the basal levels of DNA synthesis by C6 cells indicating that its effect was not due to non-specific toxicity (Figure 3).

Unlike neurons, glial cells are dividing throughout life. Furthermore, pathological conditions such as trauma transiently increase its proliferation Therefore, a knowledge of mitogenic factors that control glial cell proliferation will provide important insights into how the nervous system respond to a variety of neurological disorders. Our present results indicate that TNF-α, IL-6



(2)



(3)

Figure 2. Inhibition of cytokine-induced growth of C6 cells by L-NMMA. C6 cells were cultured in absence of any cytokine (◆, ◊) or in presence of TNF-α (500 U/ml) (■,□), IFN-γ (100 U/ml) (▲,△) or IL-6 (500 U/ml) (●,○), in medium containing 100 μM D-NMMA (closed symbols) or L-NMMA (open symbols). The number of viable cells was evaluated at the indicated times. Controls in absence of drugs were superimposed to the points with D-NMMA (not shown).

Figure 3. Effect of kinase inhibitors on cytokine-induced proliferation of C6 cells. C6 cells were cultured for 24 hrs in medium (●,○) or in presence of the indicated concentrations of TNF-α (■,□) or IFN-γ (▲, △) in presence of the indicated concentrations of H-9 (open symbols) or H-7 (closed symbols). DNA synthesis was estimated as in figure 2.

and IFN-γ induce C6 glia cells to grow faster even in presence of serum, suggesting that they play an important role "in vivo" alone or in combination with another signals in controlling the growth of glial cells. Surprisingly, TNF-β, which is thought to bind the same cell surface receptors than TNF-α and shares with it most of its biological effects (16) was unable to affect cell growth. This could indicate that there are different receptors for TNF-α and TNF-β on brain cells. Alternatively, it could mean that TNF-α and TNF-β may bind to a common receptor but may transduce different signals as it has been hypothesized (17). It is also possible that TNF-α and TNF-β bind to the same receptor but each one needs to interact with a different cell surface component in order to get the signal transduced as it is the case with several cytokine receptors (18). Moreover, there are an increasing number of reports (17-21) which show opposite effects of TNF-α and TNF-ß in many cell types, including cells of the CNS. In a recent report TNF-α, TNF-β and IL-6 but not IFN-γ were able to stimulate the growth of astrocytes (22). The reason for the discrepancies with our results are unknown but may lie on the different cells used. Our results also indicate a cooperative effect between IFN-γ and TNFα in both the stimulation of glioma growth and NO formation. Similar cooperation has been previously shown in the up-regulation of MHC class II antigens on astrocytes (6).

More interestingly, our studies also indicate that some cytokines are activating NO synthesis and using the NO generated as a mediator of their activity in glial cells. A number of recent reports indicate that astrocytes and microglial cells from the brain can be induced to produce NO (23,24). We have shown here that glia cells cultured with TNF-α and IFN-γ produced high levels of NO which were blocked by L-NMMA, a competitive inhibitor of NO synthase. Furthermore, L-NMMA, blocked the growth-inducing effect of TNF-α and IFN-γ. NO has been clearly shown to play a role as neuronal messenger (9,13) and our studies suggests that glial cells may produce it upon appropriate stimulus. Therefore, it is likely that cytokines such as TNF-α and IFN-γ acting through the NO synthase may affect glial and astrocyte proliferation and induce neuronal differentiation serving as link between CNS components. The NO produced may in turn increase cGMP levels leading to cell activation as it has been reported for glutamate receptors (13). Our results with kinase inhibitors strongly support this model. Interestingly, IL-6 does not seem to act via NO synthesis. This suggest that IL-6 affects a point in the signal transducing mechanism distal to NO synthesis.

Cytokines play an important role in the CNS. There are multiple sources of cytokines in the CNS, specially in certain disease states. During viral encephalitis, multiple sclerosis, allergic encephalitis, there are inflammatory infiltrates of activated T-lymphocytes, which could be the main source of IFN-γ,

TNF-α and IL-6 (25). In addition, cytokines such as TNF-α and IL-6 can be synthesized by astrocytes and glial cells after stimulation (2,3). Then, TNF-α acting in autocrine and paracrine fashion and may cooperate with IFN-γ in inducing glial cell proliferation stimulating gliosis, astrocytosis and neovascularization at the site of injury which are a general feature after brain damage (26). As NO has been implicated in neuronal cell death (27,28) the synthesis of NO in the brain is tightly controlled. The mechanism of regulation of NO synthesis is therefore of great importance, the understanding of which may lead to effective therapy of several diseases.

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