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Cell Surface Expression of Tumor Necrosis Factor-Alpha by Activated Rat Astrocytes

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Abstract: Astrocyte are the major glial cell type in the central nervous system (CNS), and analogous to macrophage, mediates the number of immune responses such as production of cytokines including tumor necrosis factor alpha (TNF-α) upon activation. TNF-α has been implicated in neuroimmunological disorders through killing oligodendrocytes and thus causing demyelination. It has been previously demonstrated that mitogen-activated T cells synthesized a 26 kDa precursor form of TNF-α which is bound to the surface of a membrane, and is later secreted as a 17 kDa mature version. In order to examine whether astrocytes would produce the transmembrane form of TNF-α, astrocytes were stimulated with biological stimuli and the membrane form of TNF-α was analyzed by Western blot and FACS analysis. When astrocytes are stimulated with lipopolysaccharide (LPS), IFN-γ/LPS, or IFN-γ/IL-1β, they were able to express a membrane-anchored TNF-α of approximately 26 kDa protein which was immunoreactive to an anti-TNF-α antibody, whereas unstimulated astrocytes or astrocytes treated with IFN-γ or IL-1β alone was not. Our FACS data were also consistent with the immunoblot analysis. Our result suggests that the membrane form of TNF-α expressed by activated astrocytes may cause local damage to oligodendrocytes by direct cell-cell contact and contribute to demyelination observed in multiple sclerosis (MS) and experimental allergic encephalomyelitis (EAE).
Key words: astrocytes, demyelination, transmembrane tumor necrosis factor-alpha.

Astrocyte is the major glial cell type in the CNS (Fontana *et al.*, 1987) and can be identified by an astrocyte specific marker, glial fibrillary acidic protein (GFAP) (Bignami *et al.*, 1972). Astrocytes are essential for maintaining the balanced microenvironment of the CNS. They also help maintain the integrity of the CNS environment through formation of the blood brain barrier (BBB) by interactions with its end feet processes and the endothelium of the microvasculature of the CNS (Herz *et al.*, 1990). In addition to its role in maintaining homeostasis of the brain, astrocytes have been implicated in having the capacity to function as an immunocompetent cell in the CNS (Benveniste, 1992). Astrocytes are able to respond to, and secrete a variety of cytokines including IL-1β (Lieberman *et al.*, 1989), IL-6 (Frei *et al.*, 1989; Benveniste *et al.*, 1990), TNF-α (Chung and Benveniste, 1990), TGF-β (Lindholm *et al.*, 1993), GM-CSF, and G-CSF (Malipiero *et al.*, 19 90). They also express MHC class I and class II molecules in response to virus and IFN-γ (Massa *et al.*, 1987; Fontana *et al.*, 1994).

TNF-α is a cytokine produced primarily by activated macrophages, but also by T and B lymphocytes, NK cells, astrocytes, and other non-hematopoietic tissues (Beutler, 1992). TNF-α plays pleotropic roles in immune responses, inflammation, several immunopathological disorders and apoptosis (Vasselli, 1992; Nagata and Golstein, 1995). The full length human TNF-α cDNA encodes an immature polypeptide of 233 amino acids, which consists of a 76-residue long precursor sequence and a mature TNF-α molecule of 157 amino acids (Pennica *et al.*, 1984). Mature TNF-α is approximately 17 kDa in molecular mass, and is derived from a 26 kDa integral transmembrane TNF-α molecule by proteolytic cleavage (Kriegler *et al.*, 1988). Transmembrane TNF-α has been found on activated macrophages (Kriegler *et al.*, 1988), T cells (Kinkhabwala *et al.*, 1990) and a T cell hybridoma (Browning *et al.*, 1991), and is able to kill tumor cells and virus-infected cells (Perez *et al.*, 1990). In addition to TNF-α, other hormones such as TGF-α (Bringman *et al.*, 1987) and epidermal growth factor (EGF) (Scott *et al.*, 1983) were

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reported to be expressed as a surface form.

TNF-α is of particular interest in that it has been implicated in neuropathological diseases such as MS and its animal model, EAE (Vasselli, 1992). TNF-α has a wide range of immunological functions in the CNS: these include induction of MHC class I antigens on astrocytes (Lavi *et al.*, 1988), induction of ICAM-1 on astrocytes (Shrikant *et al.*, 1994), upregulation of MHC class II molecules induced by IFN-γ and/or virus (Massa *et al.*, 1987; Vidovic *et al.*, 1990), stimulation of cytokine production such as IL-6 (Benveniste, 1990) G-CSF and GM-CSF (Malipiero *et al.*, 1990) and enhancement of permeability of endothelial cells (Brett *et al.*, 1989). Furthermore, TNF-α has been postulated to directly contribute to demyelination both by myelin damage (Selmaj and Raine, 1988) and by killing oligodendrocytes (Robbins *et al.*, 1987; Selmaj *et al.*, 1991) which synthesize and provide myelin basic protein. Astrocytes and oligodendrocytes are in close contact in terms of anatomy (Kimelberg and Norenderg, 1989). Activated astrocytes may either secrete TNF-α protein or display the transmembrane form of TNF-α, thereby killing their neighboring oligodendrocytes. It would be, therefore, interesting to know whether astrocytes would be capable of expressing the transmembrane version of TNF-α, which causes in turn the death of oligodendrocytes through direct cell-cell contact.

In this study we examined whether astrocytes would produce the transmembrane form of TNF-α upon exposure to biologic stimuli. Astrocytes were indeed able to express the membrane-anchored TNF-α when they were treated with LPS, IFN-γ/LPS and IFN-γ/IL-1β, as evidenced by immunoblot and FACS analysis. Thus, the result suggests that the transmembrane form of TNF-α expressed on the surface of activated astrocytes may be one of the factors that contribute to demyelination through direct cell-cell contact during the immunological disease state in the brain.

Materials and Methods

Materials

Rat rIFN-γ (specific activity: 4×10^6 U/ml) and human rIL-1β (specific activity: 5×10^8 U/ml) were obtained from AMGen Biologicals (Thousand Oaks, USA), and human rTNF-α (specific activity: 5.6×10^7 mg/ml) was the generous gift of Genentech, Inc. (South San Fransisco, USA). Polyclonal antibody to mouse rTNF-α ($>1.0\times10^4$ NU/mg) was purchased from Endogene (Boston, USA). Actinomycin D-manitol, MTT, and LPS (*Escherichia coli*: 0127:B8) were from Sigma (Sigma Chemical Co.; St. Louis, USA). Rat strain used in this experiment was Sprague Dawley (SD) (Prattville, USA).

Primary astrocyte cultures

Primary astrocyte cultures were established from SD neonatal rat cerebra as previously described (Chung and Benveniste, 1990). Meninges were removed before culturing. Culture medium was DMEM, high glucose formula supplemented with glucose to a final concentration of 6 g/l, 2 mM glutamine, 0.1 mM nonessential amino acid mixture, 0.1% gentamycin, and 10% FBS (HyClone; Logan, USA). Oligodendrocytes were separated from the astrocytes by mechanical dislodging after 10 days in primary culture, and then the astrocytes were obtained by trypsinization (0.25% trypsin-0.02% EDTA). The astrocytes were cultured on 100 mm dishes. In subsequent experiments, astrocytes were purified by four repetitions of trypsinization and replating to remove contaminating microglia: after such manipulation the astrocytes cultures were >99% positive for GFAP, and negative for nonspecific esterase and MAC-1 staining.

TNF-α production by astrocytes

Primary rat astrocytes were resuspended in DMEM containing 10% FBS, and plated at 1×10^6 cells/well into 6-well plates (Costa; Cambridge, USA). The plates were incubated overnight to allow recovery of the cells from trypsinization and assure adherence of the astrocytes. When the astrocytes reached confluency (1 to 2 days after plating) the original medium was aspirated off, and 2 ml of serum free DMEM was added to the wells. Astrocytes were primed with or without rat IFN-γ (100 U/ml) for 8 h, and then treated with LPS (1 μg/ml), or human IL-1β (1000 U/ml) for various periods of time. Supernatant and cell pellet were subjected to TNF biologic assay and Western blot procedure, respectively.

Measurement of TNF-α activity

TNF activity in astrocyte-derived culture supernatants was determined in a biologic assay using WEHI 164 clone 13 mouse fibrosarcoma cells (Chung and Benveniste, 1990). The actively growing cells were resuspended at a concentration of 4×10^5 cells/ml in RPMI 1640 medium containing 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 10% heat inactivated FBS, and 0.9 μg/ml actinomycin D-manitol. A total of 100 μl (4×10^4 cells/well) of this cell suspension was added per well, 100 μl of either test sample or human rTNF-α standards added and incubated at 37℃ for 16 h. Triplicate cultures were set up for each sample and standard. After this incubation, cytotoxicity was assessed using the MTT cytotoxicity assay (Espevik and Nissen-Meyer, 1986).

Western blot analysis of a membrane TNF-α

The astrocytes (6×10^6 cells) which had been treated with various stimuli were extracted with PBS, pH 7 containing 1% v/v NP-40, 2 mM PMSF for 30 min on ice. Nuclei and debris were removed by centrifugation at 12,000×*g* for 10 min. To 70 μl of cell lysates were added 20 μl of sample loading buffer. The samples were resolved on 12.5% SDS-PAGE by Laemmli method (Laemmli, 1970) and electrophoretically transferred to nitrocellulose membrane (MSI; Westbro, USA). Filters were blocked and reacted with rabbit anti-mouse rTNF-α antibody at room temperature for 1 h. After unbound antibody was washed away, the blots were incubated with 1:3000 dilution of goat anti-rabbit IgG antibody conjugated with alkaline phosphatase (Soutern Res.; Birmingham, USA) at room temperature for 30 min, and developed with substrate solution containing NBT and BICP (Sigma Chemical Co.; St Louis, USA) as manufacturer indicated.

Flow cytometry

The treated astrocytes on 6-well plates were preincubated with nonimmune rabbit serum, and then incubated with a final dilution of the rabbit anti-mouse rTNF-α antibody of 1:100 for 1 h. Cells were washed twice with PBS and then incubated with a 1:1000 dilution of fluorescein-labeled goat anti-rabbit IgG (Cappel; Durham, USA). Analyses were performed with a FACStar instrument (Beckman-Dickinson; San Jose, USA). Polyclonal antibody to mouse rTNF-α used throughout this experiment abolished the cytotoxic activity of rat TNF by approximately 95% at 10 NU (Chung and Benveniste, 1990).

Results and Discussion

We have previously demonstrated that astrocytes secreted TNF-α in response to LPS and that IFN-γ primed astrocytes to render themselves responsive to a suboptimal dose of LPS or IL-1β for subsequent TNF-α production (Chung and Benveniste, 1990). As mentioned earlier, since TNF-α is both secreted (17 kDa) and expressed as transmembrane form (26 kDa) on the surface membrane of mitogen-treated T cells (Kinkhabwala *et al.*, 1990) and activated macrophages (Kriegler *et al.*, 1988), we examined whether astrocytes could also express the membrane form of TNF-α on their surface upon stimulation. To do this, astrocytes were treated with various stimuli for 12 h, and then the secreted TNF-α activity was measured from the culture supernatant while the transmembrane form of TNF-α was assessed from cell pellet by FACS analysis. As shown in Table 1, untreated astrocytes and astrocytes treated with either IFN-γ or IL-1β alone did not produce either the secreted TNF-α or a transmembrane form of TNF-α. However, when astrocytes were treated with LPS, IFN-γ/LPS and IFN-γ/IL-1β which are known to induce TNF-α in astrocytes (Chung and Benveniste, 1990; Chung *et al.*, 1992), secretion of TNF-α was observed, and the surface form of TNF-α was detected in proportion to the amount of TNF-α secreted. Thus, both forms of TNF-α were produced from astrocytes upon stimulation.

To reveal the presence of membrane-anchored form of TNF-α, the astrocyte cultures were pretreated with IFN-γ (100 U/ml) for 8 h, and stimulated with LPS

Table 1. Membrane-bound TNF-α and secretory TNF-α produced in astrocytes

Cell Treatment	% TNF-α positive cell	Secretion of TNF-α (pg/ml)
Medium alone[a]	1.2± 0.2[e]	<5
IFN-γ (100 U/ml)[a]	2.6± 1.4	<5
IL-1β (1000 U/ml)[a]	1.4± 0.2	<5
LPS (1 μg/ml)[b]	8.2± 2.8	450± 70
IFN-γ/LPS[c]	20.3± 9.1	1974± 150
IFN-γ/IL-1β[d]	19.9± 5.9	1542± 115

[a] 12 h incubaltion.
[b] 8 h with medium, 4 h with LPS.
[c] 8 h with IFN-γ, 4 h with LPS.
[d] 8 h with IFN-γ, 4 h with IL-1β.
[e] Mean± S.D. of five experiments.

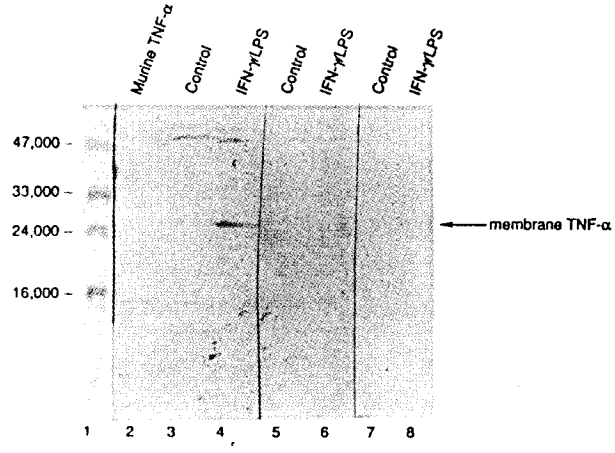

Fig. 1. Immunoblot analysis of a membrane-bound TNF-α expressed on the surface of astrocytes. The astrocytes (1×10^6 cells) on 6 well-plates were preincubated with IFN-γ (100 U/ml) for 8 h and then treated with LPS (1 μg/ml) for 4 h (lane 4, 6, 8). The total cell lysates were subjected to Western blot analysis with rabbit anti-mouse TNF-α antibody (lane 2-4) and unimmune rabbit serum (lane 5, 6). No serum was used for analysis (lane 7, 8). Lane 2 indicates recombinant mouse TNF-α molecule (10 ng) for positive control.

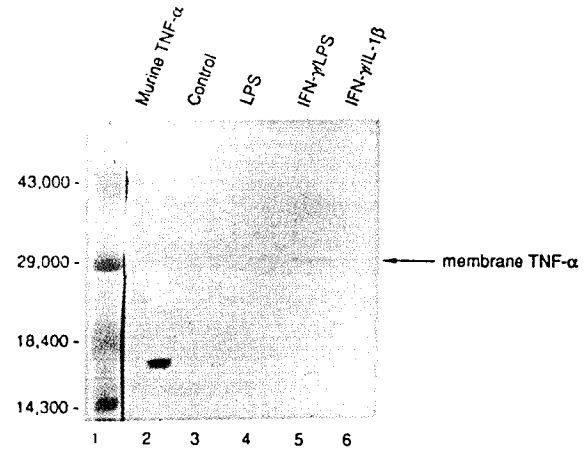

Fig. 2. Immunoblot analysis of a membrane-bound TNF-α expressed on astrocytes treated with various stimuli. The astrocyte cultures were pretreated with or without IFN-γ (100 U/ml) for 8 h and then treated with medium (lane 3), LPS (1 μg/ml) (lane 4, 5) and IL-1β (lane 6) for 4 h. Total cell lysates were subjected to Western blot analysis with rabbit anti-mouse TNF-α antibody.

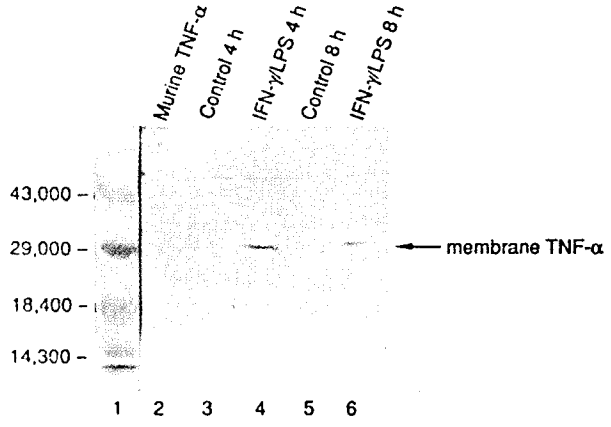

Fig. 3. Kinetic analysis of IFN-γ/LPS treatment on astrocyte membrane-bound TNF-α. The astrocyte cultures were pretreated with IFN-γ (100 U/ml) for 8 h and then treated with LPS (1 μg/ml) for 4 h (lane 4) and 8 h (lane 6). Lane 3 and 5 represent cell lysates from unstimulated astrocytes for 4 h and 8 h, respectively. Immunoblot analysis was carried out using rabbit anti-mouse TNF-α antibody.

(1 μg/ml) for 4 h. Then, the cell lysates were subjected to Western blot analysis. The blot was incubated with rabbit anti mouse rTNF-α antibody (lane 2-4), non-immune rabbit serum (lane 5 and 6), and no serum (lane 7 and 8) (Fig. 1). As a positive control, mouse rTNF-α was used and was reactive to the anti-mouse rTNF-α antibody (lane 2). The same antibody specifically detected 26 kDa protein only in cell lysate from IFN-γ /LPS-treated astrocytes (lane 4), but the protein species did not appear from unstimulated astrocytes (lane 3). Also, non-immune rabbit serum failed to react to any protein both from stimulated and unstimulated cells (lane 5 and 6). Next, we examined whether other stimuli could induce expression of membrane form of TNF-α. As shown in Fig. 2, unstimulated astrocytes could not induce (lane 3) but LPS, IFN-γ/LPS, and IFN-γ/IL-1β induced membrane form of TNF-α (Lane 4-6). Furthermore, astrocytes were pretreated with IFN-γ for 4 h and subsequently treated with LPS for 4 h and 8 h, and the culture supernatant and cell pellet were assayed for biologic activity (Table 2) and immunoblot analysis (Fig. 3), respectively. The membranous TNF-α was expressed at a relatively higher level at 4 h than at 8 h after LPS treatment as biologic activity of TNF-α was. Thus, immunoblot analysis was consistent with FACS data and biologic activity: unstimulated astrocytes and IFN-γ- or IL-1β-treated astrocytes did not either secrete TNF-α or express detectable membranous TNF-α, whereas astrocytes which had been treated LPS, IFN-γ/LPS, and IFN-γ/IL-1β produced both secretory and membrane-bound TNF-α. This data indicated that stimulated astrocytes display a membrane form of TNF-α with approximate molecular mass of 26 kDa.

Table 2. Kinetic analysis of IFN-γ/LPS treatment on astrocyte TNF-α secretion

Cell Treatment	TNF-α (pg/ml)
Control (4 h)	<5
IFN-γ[a]/LPS[b] (4 h)	2625± 205
Control (8 h)	<5
IFN-γ[a]/LPS[b] (8 h)	1529± 89

[a]IFN-γ (100 U/ml), preincubation for 8 h.
[b]LPS (1 μg/ml).

This study demonstrated that astrocyte was able to express both secretory and membrane-anchored TNF-α *in vitro* in response to biologic stimuli. The physiological significance of activated astrocytes to express the surface form of TNF-α is not clear. Astrocytes are in very close proximity *in vivo* to oligodendrocytes which produce myelin basic protein and are responsible for myelination of surrounding neurons in the CNS (Kimelberg and Norenderg, 1989). As described earlier, some lymphoid cell types express a transmembrane form of TNF-α which is able to kill the target cells through direct cell-cell contact (Perez *et al.*, 1990), and TNF-α has been shown to be directly cytotoxic to oligodendrocytes (Robbins *et al.*, 1987; Selmaj *et al.*, 1991). During neuroimmunopathological state of CNS, various types of cell may produce TNF. These include T cells, macrophages, microglia and astrocytes (Benveniste, 1992). Our result demonstrated, based on the Western blot and FACS analysis, that membrane-anchored TNF-α is detectable on the surface of astrocytes which have been exposed to LPS, IFN-γ/LPS, and IFN-

γ/IL-1β. Secreted TNF-α by astrocytes is likely to be involved in augmentation of immune responses, such as in antigen presentation cytokine production, and cell killing in paracrine fashion, whereas the surface form of TNF-α may be associated with local damage by killing oligodendrocytes and causing demyelination. The novel observation that stimulated astrocytes can express a surface bound form of TNF-α suggests that direct cell-cell contact between astrocytes and oligodendrocytes could lead to oligodendrocyte damage, thereby contributing to demyelination.

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