Tumor Necrosis Factor α Induces Adhesion Molecule Expression on Human Fetal Astrocytes

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Summary

Leukocyte adhesion molecules on endothelial cells of the blood-brain barrier may participate in the entry of leukocytes into the central nervous system. Because astrocytes are also a component of the blood-brain barrier and have been associated with inflammation, we studied the ability of astrocytes to express leukocyte adhesion molecules using Northern blot and immunocytochemical techniques. Astrocytes treated with the proinflammatory cytokine tumor necrosis factor α (TNF) expressed messenger RNA for the adhesion molecules E-selectin, vascular cell adhesion molecule 1, and intercellular adhesion molecule 1, as well as their corresponding proteins. In addition, TNF-treated astrocytes expressed a monocyte adhesion protein identified by our laboratory, recognized by the monoclonal antibody IG9. These results indicate that under inflammatory conditions in the central nervous system, such as multiple sclerosis and acquired immune deficiency syndrome, astrocyte expression of adhesion molecules may facilitate the migration of leukocytes and contribute to the disease process.

Leukocyte adhesion molecules participate in the rolling, adhesion, and extravasation of leukocytes across the vascular endothelium (1, 2). Upregulation of adhesion molecule expression on endothelial cells (EC) has been demonstrated in immune-mediated diseases (3–9). Because immune cells are detected within the central nervous system (CNS) in diseases such as multiple sclerosis (10) and AIDS (11), adhesion molecules may facilitate the transmigration of leukocytes through the blood-brain barrier (BBB) and into the CNS, thereby contributing to the neuropathology of these diseases (5, 12).

In addition to EC, astrocytes are a structural and functional component of the BBB (13–15). Astrocyte processes are found in close apposition to the abluminal surface of the endothelium and have been shown to contribute to the formation of the BBB (14, 15). Astrocytes can also participate in immune-mediated events (16–21). For example, in animal models of autoimmune disease as well as in tissue culture, astrocytes can present antigen to MHC-restricted T lymphocytes (16–21). Also, astrocytes can respond to cytokines by upregulating their MHC antigen expression (19–21) and by secreting additional cytokines (21, 22). Recently, several studies have shown that astrocytes can be induced to express the leukocyte adhesion molecule, intercellular adhesion molecule 1 (ICAM-1) (20, 23–25). Because neurologic dysfunction is a prominent finding in pediatric AIDS and the precise pathogenic mechanism is as yet undefined, we determined if human fetal astrocytes can express a repertoire of leukocyte adhesion molecules in response to the proinflammatory cytokine, tumor necrosis factor α (TNF).

Materials and Methods

Source of Fetal Tissue. The present study is part of an ongoing research protocol that has been approved by the Albert Einstein College of Medicine Committee on Clinical Investigation and the City of New York Health and Hospitals Corporation. Informed consent was obtained from all participants. Fetal tissues were obtained at the time of elective termination of intrauterine pregnancy from otherwise normal healthy females. Gestational age was determined by multiple parameters including the date of the last menstrual period by history, uterine size by bimanual and abdominal examination, ultrasonography using predominantly the maximum biparietal diameter, and, postabortal, by measurement of the fetal foot length (26). The tissues used in this study were obtained from 21–23 wk-old fetuses.

Cell Culture. Astrocyte cultures were prepared according to the protocol of McCarthy and de Vellis (27). Briefly, human fetal CNS tissue was separated from the meninges, minced, and digested in 0.25% trypsin (Gibco Laboratories, Grand Island, NY)/0.1% collagenase (Sigma Chemical Co., St. Louis, MO). The resulting cell suspension was serially filtered through sterile 120- and 80-μm nylon mesh filters (Tetko, Elmsford, NY) and pelleted at 900 rpm. Cul
tures were established in RPMI supplemented with 10% FCS (Whittaker M. A. Bioproducts, Walkersville, MD). To enrich for astrocytes, cultures were passaged after trypsinization. Astrocytes were treated with 400 U/ml TNF (endotoxin <1 ng/5 x 10^6 U TNF) (R&D Research) for 5 or 16 h.

**Northern Blot Analysis.** Total RNA was extracted from three sets of untreated and TNF-treated astrocyte cultures according to the method of Chomczynski and Sacchi (28). RNA samples (20 µg) were denatured and fractionated on 1% agarose gels prepared in 6.5% formaldehyde/1× MOPS buffer. After electrophoresis, RNA was transferred to a nitrocellulose filter and hybridized with the following 32P random primer-labeled (Amersham Corp., Arlington Heights, IL) cDNA probes: E-selectin (29) (6.0-kb cDNA in pCDN-Tm+4 vector), vascular cell adhesion molecule 1 (VCAM-1) (30) (6.5-kb insert in pCDN vector) (both provided by Dr. Walter Newman, Otsuka America Pharmaceuticals, Rockville, MD), or ICAM-1 (31) (4.8-kb insert in CDM8 vector, provided by Dr. Timothy Springer, Center for Blood Research, Harvard Medical School, Boston, MA). To demonstrate equivalent loading of RNA, filters were stripped of radiolabeled probe by boiling and rehybridized with a cDNA probe to human 18S RNA (32). Previous studies have used 18S RNA expression as a control for gene expression in astrocytes (33).

**Immunocytochemistry.** Astrocyte cultures were washed, fixed in cold absolute methanol for 10 min, rinsed, and treated with a solution of 5% nonfat milk in PBS to block nonspecific antibody binding. Astrocytes were then incubated with one of the following primary antibodies for 2 h: anti-Glial Fibrillary Acidic Protein (GFAP) (IgG1, 1:200; Boehringer Mannheim Biochemicals, Indianapolis, IN), anti-ELAM-1 (IgG1, 1:100; Biodesign International, Kennebunkport, ME), anti-VCAM-1 (IgG1, F(ab')2, 2.9 mg/ml, 1:200; Dr. Walter Newman, Otsuka America Pharmaceuticals), IG9 (IgG3, ascites, 1:2,000), anti-ICAM-1 (1:100; Biodesign International), and anti-factor VIII-related antigen (IgG1, 1:100; Dako Corp., Santa Barbara, CA). After washing the cells, peroxidase-coupled, class-specific secondary antibodies were added. Immunoreactivity was visualized using diaminobenzidine (Sigma Chemical Co.). Immunocytochemical results are representative of six separate experiments using cultures derived from different specimens.

**ELISA.** Astrocytes cultured in 96-well dishes were treated with TNF for 4, 16, 24, or 48 h and thereafter fixed in cold absolute methanol. Subsequently, all steps were as detailed above, except that tetramethylbenzidine (TMB; Boehringer Mannheim Biochemicals) was used as a chromagen. After incubation with a peroxidase-coupled antibody, cultures were washed, and 100 µl TMB (0.1 mg/ml)/H2O2 (0.3%) solution was added to each well. The reaction was stopped by the addition of 25 µl 8 N sulfuric acid and the absorbance measured at OD 450 nm using an EIA reader (Bio-Rad Laboratories, Richmond, CA). Values represent the average of quadruplicates ±1 SD. The histogram presented in Fig. 4 is representative of five experiments each using astrocytes from different fetuses.

**Results and Discussion**

Cultures enriched for astrocytes by serial passage were found to be >95% GFAP⁺ (Fig. 1 a). Two morphologically dis-
distinct populations could be detected: a flat attenuated form rich in GFAP+ cytoskeletal fibers and a multipolar form with long GFAP+ processes. TNF treatment for either 5 or 16 h did not change this immunoreactivity in either cell population.

E-selectin (34, 35) is a member of a family of inducible cell adhesion proteins whose carbohydrate ligands (36) are expressed by neutrophils, monocytes, and a subset of memory T cells. To test for the expression of E-selectin by astrocytes, cultures were treated with TNF for 5 h. These cultures expressed E-selectin message (Fig. 2) and protein (Fig. 3 a). Similar results were obtained after TNF treatment of astrocyte cultures for 16 h. Untreated astrocytes did not express E-selectin message (Fig. 2) or protein (Fig. 1 c).

The expression of an adhesion molecule that was recently identified in our laboratory (3, Calderon et al., manuscript submitted for publication) was also studied. mAb IG9 inhibits monocyte adhesion to TNF-treated EC. The 105-kD IG9 protein is not expressed on untreated EC but can be induced on the cell surface by TNF, IL-1, and LPS. Maximal expression is detected 4–9 h after stimulation and is no longer detectable after 48 h. Preclearance of radiolabeled TNF-treated EC surface protein lysates by immunoprecipitation with anti-E-selectin or anti-VCAM-1 antibodies does not diminish the levels of immunoprecipitable IG9 protein. These and other results (Calderon et al., manuscript submitted for publication) suggest that the IG9 protein is a monocyte adhesion molecule distinct from E-selectin and VCAM-1. In the present study, astrocytes were treated with TNF for 5 or 16 h and assessed for IG9 immunoreactivity. IG9 protein expression was detected in TNF-treated astrocytes (Fig. 2 b) but was absent in untreated cultures (Fig. 1 d). Unlike the other adhesion molecules examined, IG9 protein appeared to be differentially expressed on distinct populations of astrocytes as there was more immunoreactivity detected in the flatter population of cells when compared with the multipolar form.

VCAM-1 (37, 38) is a member of the Ig supergene family. Its ligand, very late antigen 4, is expressed on lymphocytes and monocytes (39). Untreated astrocytes expressed low levels of VCAM-1 message (Fig. 2) and few cells were positive by immunocytochemistry (Fig. 1 e). As shown in Fig. 2, Northern blot analysis revealed detectable VCAM-1 message after 5 h of TNF stimulation; however, no immunoreactivity for VCAM-1 protein was detected at this time point. In contrast, after treatment with TNF for 16 h, astrocytes expressed detectable VCAM-1 message and protein (Fig. 3 c).

ICAM-1 is also a member of the Ig supergene family whose ligand, lymphocyte function antigen 1, is expressed on all leukocytes (31, 40). Previous studies have shown that TNF-treated astrocytes express ICAM-1 (23, 24). To confirm that human fetal cells would respond to TNF in a similar manner, fetal astrocytes were cultured in the presence of TNF for 5 or 16 h and tested for ICAM-1 expression. Untreated astrocytes did not express ICAM-1 message (Fig. 2) or protein (Fig. 1 f). As shown in Figs. 2 and 3 d, astrocytes expressed detectable ICAM-1 message and protein after 5 h of TNF stimulation. Both message and protein were also detected at 16 h.

To determine the kinetics of adhesion molecule expression by astrocytes, a cell-based ELISA was performed. As shown in Fig. 4 and confirmed by immunocytochemical data presented above, E-selectin, IG9, and ICAM-1 proteins were detected by 5 h after TNF stimulation, whereas a significant increase in VCAM-1 expression was not detected until 16 h after TNF stimulation. Maximal IG9 and E-selectin expression was detected at 4 h, whereas VCAM-1 and ICAM-1 reached peak expression at 16 h. In addition, IG9 expression was quantitatively lower than E-selectin, VCAM-1, and ICAM-1. These latter kinetic data also confirm the immunocytochemical observations.

The data reported in this communication support two conclusions about leukocyte adhesion molecule expression by human fetal astrocytes. The first is that this cell type can be induced to express adhesion molecules. Previous studies using cultured astrocytes (20, 23, 24) have demonstrated ICAM-1 expression in vitro. In addition, studies examining in vivo expression of adhesion molecules in CNS inflammatory diseases have suggested that astrocytes may also express adhesion molecules (5, 12). Preliminary studies in our laboratory using double immunofluorescence colocalized GFAP and VCAM-1 in active multiple sclerosis lesions. Immunocytochemical and Northern blot analyses presented in the present

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**Figure 2.** Northern blot analysis of adhesion molecule expression by human fetal astrocytes. RNA from untreated or 5-h TNF-treated human fetal astrocytes was subjected to electrophoresis through a 1% agarose gel, transferred to nitrocellulose, and probed using a 32P-labeled cDNA probe for the indicated adhesion molecules. Untreated astrocytes expressed no E-selectin or ICAM-1 mRNA, whereas low levels of VCAM-1 message were detected. TNF-treated astrocytes expressed message for all three adhesion molecules. Filters were stripped of adhesion molecule probes and rehybridized with an 18S RNA probe to demonstrate equivalent RNA loading.
Figure 3. Immunocytochemical analysis of adhesion molecule expression. Methanol-fixed, TNF-treated (5 h: E-selectin, IG9, and ICAM-1; 16 h: VCAM-1) astrocytes were tested for adhesion molecule immunoreactivity. TNF-treated astrocytes expressed E-selectin (a), IG9 (b), VCAM-1 (c), and ICAM-1 (d) proteins.

Figure 4. Kinetic analysis of adhesion molecule expression. Untreated or TNF-treated astrocytes were tested for adhesion molecule expression. Astrocyte cultures were treated with TNF and fixed as described above. Immunoreactivity was detected using peroxidase/TMB and analyzed at OD 450 nm. Data (±1 SD) are representative of five experiments, each using different astrocyte cultures.

The results presented in this report further implicate astrocytes in the immune response within the CNS. Accordingly, once monocytes and lymphocytes have crossed the BBB, their migration to sites of inflammation within the CNS parenchyma may be facilitated by astrocyte expression of adhesion molecules. In diseases such as AIDS or multiple sclerosis, where immune cells are detected within the CNS, adhesion molecule expression by astrocytes may function in such a manner. Current experiments designed to examine the in vivo expression of adhesion molecules by astrocytes during pathologic conditions should help to clarify the role of astrocytes in CNS inflammatory disease.
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