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CHAPTER 20

A RT-PCR study of gene expression in a mechanical injury model

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Introduction

Astrocytes make up a substantial proportion of the central nervous system (CNS) and participate in a variety of important physiologic and pathologic processes. They are characterized by vigorous response to diverse neurologic insults and induction of the glial fibrillary acidic protein (GFAP) gene, a feature that is well conserved across a variety of different species. The prominence of astroglial reactions in various diseases, the rapidity of the astroglial response, and the evolutionary conservation of reactive astrogliosis indicate that reactive astrocytes fulfill important functions of the CNS. Yet, the exact role reactive astrocytes play in the injured CNS has so far remained elusive. The astroglial response can be detected within one hour of a focal mechanical trauma (Mucke et al., 1991). Intense reactive astrogliosis is seen: in AIDS dementia; a variety of other viral infections; prion associated spongiform encephalopathies; inflammatory demyelinating diseases; acute traumatic brain injury; neurodegenerative diseases such as Alzheimer's disease (Eng et al., 1992).

The activated astroglia may benefit the damaged nervous system by participating in several important biologic processes: regulation of neurotransmitter levels; repair of the extracellular matrix; control of the blood–CNS interface; control of transport processes; and trophic support of other CNS cells. However, gliosis may produce pathological effects by interfering with the residual neuronal circuits, by preventing remyelination, or by inhibiting regeneration. It is therefore important to identify the essential molecular mechanisms which activate metabolic responses in astrocytes in CNS disease and injury. These signals may result from anoxia due to disruption of the blood supply; dilution of inhibitory "chalones" around the injury site due to edema; changes in the ionic and molecular composition of the CNS extracellular fluid after disruption of the blood–brain barrier; release of growth factors and cytokines from non-CNS cells such as macrophages, T- and B-cells which may infiltrate the lesion; and loss of ionic coupling and release from cell density-dependent inhibition of growth due to increase in extracellular space. The signals also include factors from activated endothelial cells and microglia at the site of the lesion and secretion of mitogenic and trophic factors from degenerating neurons and oligodendroglia. To understand astrogliosis, it is

necessary to identify the essential molecular mechanisms by following the gene and protein expression after astrocyte injury. The development of a highly sensitive reverse transcriptase–polymerase chain reaction (RT-PCR) method now allows us to measure the mRNAs of growth factors, cytokines, enzymes and proteins that are specifically induced by injury (Murphy et al., 1993a).

At present, the majority of studies have used astrocyte and glioma cell cultures with few *in vivo* studies to confirm the *in vitro* results. While reactive astrocytes in adult brains and primary astrocytes in cell culture differ in many respects and results obtained *in vitro* and *in vivo* may often not agree, astrocytes in culture can be activated and express all of the important characteristics of astrogliosis after injury in a system without complications by neural, hormonal and hemodynamic factors (Yu et al., 1993a). It is evident that astrocytes in culture can produce many molecules in response to various agents, however, the current challenge is the identification of those factors which induce specific astrocytic responses *in vivo* and the determination of how these responses contribute to regeneration and degeneration of the CNS. Identification of those factors that promote and those that inhibit regeneration will allow one to devise therapeutic protocols to treat CNS injury. These treatments could employ antisense oligonucleotides, antibodies, growth factors, enzymes, pharmaceutical drugs, and gene therapies.

Reactive astrocyte

Using induction of GFAP expression as the main indicator of astroglial activation, *in vivo* and *in vitro* studies have reported that activated astrocytes express antigen presentation, adhesion molecules, cytokines, growth factors, early response genes, eicosanoids, and enzymes (Eddleston and Mucke, 1993) and summarized below.

1. *Activated astrocytes express*: antigen presentation: major histocompatibility complex (MHC class I, MHC class II); calcium binding protein, S-100β; receptors: epidermal growth factor (EGF), tissue factor, tumor necrosis factor (TNFα), transferrin; transport: apolipoprotein E, transferrin; cytoskeleton: intermediate filament associated protein (IFAP), microtubule-associated protein (MAP2), vimentin, glial fibrillary acidic protein (GFAP) (Eng and Lee, 1995); epitopes: J-31, LN-1, M1, M22, x-hapten (Le^x); miscellaneous: ab crystallin, C3, factor B, GC, GD3 ganglioside, membrane cofactor protein (MCP) (CD46), sulphated glycoprotein (SGP-2), peripheral type benzodiazepine binding site ("w3"-PTBBS).

2. *Activated astrocytes express adhesion molecules*: Cluster designation (CD44), chondroitin-6-sulfate proteoglycan, embryonic neural cell adhesion molecule, HNK-1, glial hyaluronate adhesion molecule, heparin sulfate proteoglycans, intercellular adhesion molecule, Laminin, Tenascin (cyclotactin), and Thrombospondin.

3. *Activated astrocytes express cytokines and growth factors*: fibroblast growth factor (aFGF, bFGF), Endothelin 1, granulocyte (macrophage)-colony stimulating factor (G-CSF, G(M)-CSF), interferon (IFNα, IFNα/β, IFNβ, IFN γ), insulin-like growth factor (IGF-1), interleukin (IL, IL-1α, IL-1β, IL-6), nerve growth factor (NGF), CNTF, NT3, transforming growth factor (TGFα, TGFβ-1, TGFβ-3), tumor necrosis factor (TNFα), lymphotoxin (LT), macrophage inflammation protein 1α (MIP-1α), and leukemia inhibitory factor (LIF).

4. *Activated astrocytes express early response genes*: AP1, c-Fos (TIS 28), c-Jun, hsp68/70/72, nerve growth factor (NGF1α) (TIS 8, egr-1, krox-24, zif 268), NGF1b (nur 77, TIS 1), TIS 10, TIS 11, and TIS 21.

5. *Activated astrocytes express eicosanoids*: Leukotriene B4, Leukotriene C4, Prostaglandin E, Prostaglandin E2, Thromboxane A2, and Thromboxane B2.

6. *Activated astrocytes express enzymes*: car-

bamyl phosphate synthetase II, aspartate transcarbamylase/dihydroorotase CAD multidomain complex, calcium (Ca^{2+}) ATPase, Carbonic anhydrase II, Glutamine synthetase, Glutathione transferase Y_b, Protein kinase Ca, Protein kinase Ca/b, Calpain I, Carboxypeptidase E, Cathepsin B, Cathepsin D, Protease inhibitors: α1-antichymotrypsin(α1-ACT), amyloid β protein precursor (APP), protease nexin (PNII), plasminogen activator inhibitor (PAI), PNI, glia derived nexin (GDN), and tissue inhibitor of metalloprotease (TIMP) related protein.

Cytokines

Numerous studies have demonstrated that cytokines are present in the CNS during neurological diseases. The production of these cytokines has not only been attributed to the activated lymphoid and mononuclear cells that infiltrate the CNS as a result of disease, but also to the resident glial cells such as astrocytes and microglia. Cytokines present in the CNS during disease states and their cellular localization are: IL-1 (astrocytes and microglia); IL-2 (activated T cells); IL-6 (astrocytes); Interferon-γ (INF-γ) (activated T cells); tumor necrosis factor-α (astrocytes, macrophages); LT (T-cells, macrophages); and TGF-β (astrocytes, microglia, macrophages).

Several *in vitro* and *in vivo* studies have been conducted to investigate whether the cytokines detected in the CNS contribute to the activation/proliferation of astrocytes. The availability of recombinant cytokines has facilitated the studies on effects of cytokines on glial cell function. The effects of recombinant cytokines such as IL-1, INF-γ, TNF-α and IL-6 on glial cell function as well as various stimuli that induce the production of these cytokines in glial cells have recently been reviewed by Benveniste (1993) and summarized below.

IL-1 has been shown to induce: proliferation of neonatal astrocytes and human astroglioma cells; IL-6 expression by neonatal astrocytes and human astroglioma cells; TNF-α expression in conjunction with INF-γ by neonatal rat astrocytes and human astroglioma cell lines; G-CSF and GM-CSF expression in human astroglioma cell lines, increase ICAM-1 expression on human fetal and adult astrocytes and enhance expression of C3 complement component in rat astrocytes and human astrogliomas. In addition, human glioblastoma cells have been shown to constitutively secrete IL-1 but astrocytes and microglia produce IL-1 only in response to lipopolysaccharide (LPS) stimulation.

INF-γ increases: class I MHC expression in primary astrocytes and microglia; class II MHC expression on primary astrocytes, human glioma cells and microglia; ICAM-1 expression on human fetal and adult astrocytes; TNF-α receptor expression on primary astrocytes, human glioma cells and microglia. INF-γ also primes rat astrocytes for TNF-α and IL-6 production, and enhances expression of complement C3 in primary astrocytes and human glioma cells.

TNF-α increases class I MHC expression on primary astrocytes; enhances class II MHC expression induced by IFN-γ or virus on primary astrocytes; induces ICAM-1 expression on human fetal and adult astrocytes; induces proliferation of adult astrocytes and human astroglioma cell lines; induces IL-6 production in primary astrocytes; enhances expression of C3 complement component in rat astrocytes and human astroglioma cells. It has been shown that astrocytes make TNF-α in response to LPS, virus, IFN-γ/IL-1 while human astroglioma cells make TNF-α in response to IL-1, PMA, calcium ionophore. LPS and IFN-γ stimulate TNF-α production in microglia. Astrocytes also express high affinity TNF-α receptors.

IL-6 induces proliferation of astrocytes and enhances NGF production by primary astrocytes. Astrocytes make IL-6 in response to LPS, IL-1, TNF-α, IFN-γ/IL-1, virus, and calcium ionophore. Human glioma cells make IL-6 in response to IL-1, and microglia make IL-6 in

response to macrophage colony stimulating factor (M-CSF) and virus.

Neurotrophic factors

Cultured astrocytes express a wide range of molecules with neurotropic properties, including conventional neurotrophins such as NGF and NT-3 as well as proteins with multiple activities-the pleiotrophins such as FGF, CNTF, and S100B. *In vitro* studies on the regulation of these factors in astrocytes have focused on astrocyte-derived NGF due to the availability of reagents to detect this factor. NGF is regulated by a disparate group of exogenous agents that include β-adrenergic agonists, phorbol esters, cytokines, and growth factors. These findings suggest that astrocytes may be involved in the expression of neurotrophic factors *in vivo*. The pleiotrophins are expressed in astrocytes *in vivo* — FGF, S100B, and CNTF — but none possess a signal sequence and, thus, cannot be released by conventional means. The mechanism(s) of their induction and release are still unknown. The expression and release of a wide range of neurotrophic factors by astrocytes in the adult CNS appear to coincide more with a traumatic event rather than with constitutive expression. Severe trauma, resulting in neuronal degeneration, causes a sustained rise in neurotrophins and pleiotrophins at the wound site, in the glial scar, and, more specifically, in reactive astrocytes (Rudge, 1993).

Scratch wound model

The aim of this study was to investigate the possibility of utilizing antisense oligonucleotides in controlling the response of astrocytes after mechanically induced injury. We scratched primary astrocyte cultures prepared from newborn rat cerebral cortex with a plastic pipette tip which resulted in areas free of astrocytes. This injury model was used to study the astrogliotic responses in culture. Injured astrocytes became hyperplastic, hypertrophic, and had an increased GFAP content. These observations demonstrate that injured astrocytes in culture are capable of becoming reactive and exhibit gliotic behaviors in culture without neurons. Electron microscopy showed the number of filaments increased in injured astrocytes. The density of fibrils is specially high in the cytoplasm of cells along the scratched edge. We studied the changes in the components of these fibrils by double immunostaining for GFAP and vimentin in injured astrocytes. The control culture contains cells stained with vimentin and/ or GFAP. This indicated that these cultures contained a mixture of astrocytes which were at different maturational stages. There were many cytoplasmic processes along the edge of the scratch immediate after scratching. These processes may be the remains from the retracted cell bodies along the scratch. After a few hours, these cytoplasmic processes became hypertrophic and the staining for these cytoskeleton proteins were stronger than cells away from the scratch. The extension of the astrocytic processes into the denuded area after 30 hours is shown in Fig. 1. The cytoskeletal response was the same whether the cells stained for only GFAP or vimentin. This indicates that astrocytes at different stages of maturation respond to injury by sending out hypertrophic processes containing an increase of GFAP. In astrocytes positively stained for both cytoskeleton proteins, most of the time the vimentin staining was located close to the nuclei. The increase in GFAP content in injured astrocytes could be inhibited by incubating the scratched culture with Lipofectin™ complexed with 3′ or 5′ antisense oligonucleotides (20 nt) in the coding region of mouse GFAP. The scratch model provides a simple system to examine in more detail the mechanisms involved in triggering glial reactivity and many of the cellular dynamics associated with scar formation. Antisense oligonucleotide treatment could inhibit the GFAP synthesis in injured astrocytes, and may be applicable in modifying scar formation in CNS injury *in vivo* (Yu et al., 1993a,b,c).

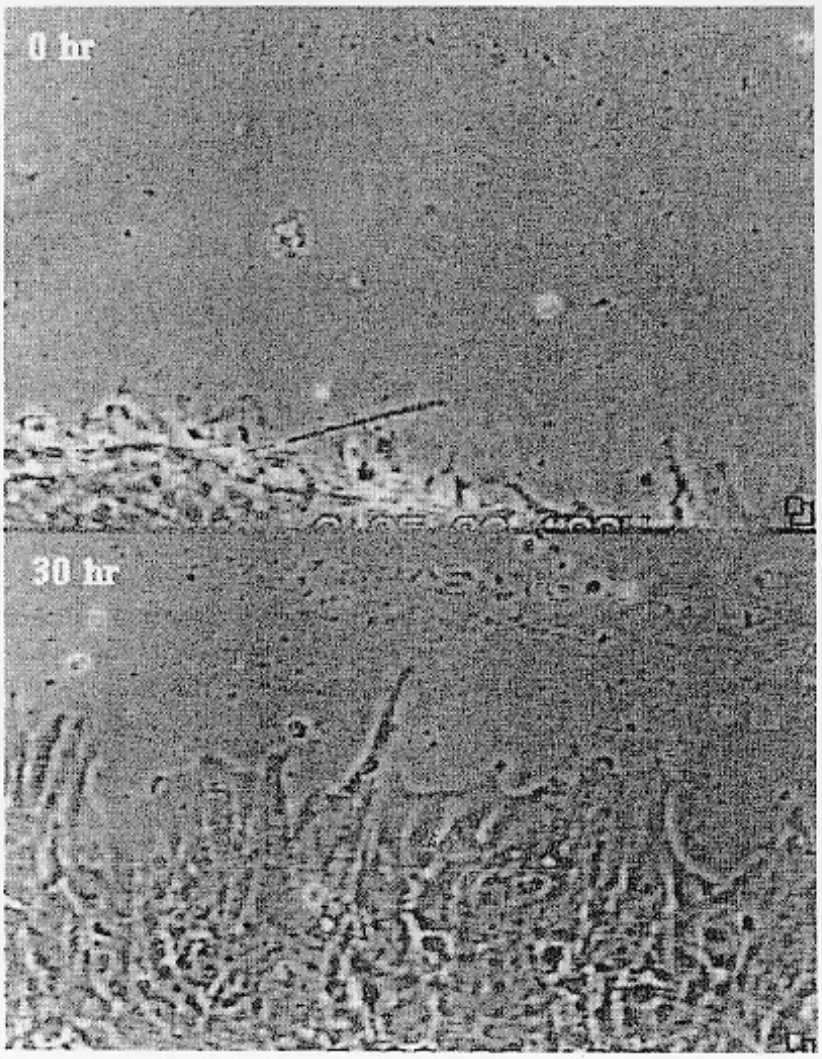

Fig. 1. Time-lapse video micrographs of astrocytes in culture after scratch injury. The videos were taken from the same field. (A) With fresh medium immediately following the scratch; and (B) with no change of culture medium 30 hours after the scratch.

RT-PCR quantification of mRNA

A reverse transcription/polymerase chain reaction technique has been developed for comparison of mRNA levels among samples (Murphy et al., 1993a). Briefly, reverse transcription is performed on total cellular RNA using AMV reverse transcriptase. After reverse transcription, the resulting cDNA is amplified using PCR with primers specific for the particular cytokine of interest. The PCR products are transferred to a nylon membrane in a slot blot apparatus, hybridized with a ^{32}P radiolabelled probe, and the hybridization signal detected with autoradiography. Autoradiographs are quantified on a Pharmacia LKB laser densitometer. This instrument provides a measure of signal intensity for each slot in the array, which is expressed in

arbitrary densitometer units. We have found that scan and re-scan variation is less than one percent.

Samples from treated and control cultures are compared by performing the PCR reaction within the exponential range, which assures that the result is proportional to the starting material. For each total RNA sample, multiple PCR reactions are performed using serial dilutions of the corresponding cDNA. Signal from these dilutions is quantified, and if they fall in a straight line on log–log paper, the amplification occurred in the exponential range. Then, equivalent dilutions of cDNA from different experimental conditions can be compared, if all amplifications occurred exponentially. The expression of a battery of control genes, including HPRT, β-actin, G3PDH, and dihydrofolate reductase, are compared among experimental conditions to assure equal RNA loading. The advantage of this method over Northern blotting is greater sensitivity, because of the tremendous amplification power of PCR. The method is also highly specific, as the PCR primers are specific for a given sequence of cDNA, PCR products are checked for predicted size on agarose gels, and the products are further identified by hybridization with a probe internal to the two primer sites.

Results and discussion

We have used the RT-PCR method to study cytokine expression in astrocytes in response to exogenous agents and mechanical injury. Recently we showed by quantitative RT-PCR that TNFα/IL-1β induced IL-6 in mouse astrocyte cultures (Murphy et al., 1993a). IL-6 belongs to a family of neuroactive cytokines which includes leukemia inhibitor factor (LIF) and ciliary neurotrophic factor (CNTF) (Patterson, 1992). RT-PCR has been used to demonstrate that the cholinergic differentiation factor/leukemia inhibitory factor (CDF/LIF) and CNTF induce mRNAs for choline acetyltransferase, somatostatin, substance P, vasoactive intestinal peptide, cholecystokinin, and enkephalin in cultured sympathetic neurons (0.4–1.6 ng/ml for as few as 3000 cells) (Fann and Patterson, 1993). These data suggest that CDF/LIF and CNTF may share receptor subunits and signal transduction pathways (Ip et al., 1992). We have also found that LPS, PMA, TNFα, and IL-1β induce LIF in cultured mouse astrocytes but not in the immortalized microglial cell line (BV-2) provided by Bocchini et al. (1992) (Murphy et al., 1995a).

TNF-α is a pluripotent cytokine with effects on a wide variety of cells. Although TNF-α has cytotoxic effects on certain tumor cells, it has been reported that TNF-α causes proliferation of astrocytes and a decrease in GFAP mRNA (Selmaj et al., 1991). TNF-α has been reported to be increased in AD, MS, HIV encephalitis, and other neuropsychiatric conditions. Using RT-PCR and the enzyme-linked immunoassay procedure, we examined the expression of GFAP in primary and secondary mouse astrocyte cultures and in the human U373 astrocytoma cell line after treatment with TNF-α. Treatment with TNF-α for 72 hours resulted in a decrease in GFAP and its encoding mRNA. Thymidine uptake studies demonstrated that U373 cells proliferated in response to TNF-α, but primary neonatal astrocytes did not. TNF-α induced an increase in total cellular protein in both U373 cells and astrocytes. Treatment of astrocytes and U373 cells for 72 hours with bFGF also induced a decrease in GFAP and an increase in total protein, demonstrating that this effect is not specific for TNF-α. The decrease in GFAP detected after TNF-α treatment is most likely due to dilution by other proteins and mRNAs which are synthesized rapidly in response to cytokine stimulation (Murphy et al.,1993b).

Macrophage inflammatory protein 1 alpha (MIP-1α) is a member of a large superfamily of inflammatory cytokines. Proinflammatory stimuli have been shown to induce expression of MIP-1α by macrophages. MIP-1α also activates macrophages, and is chemokinetic for

neutrophils. A wide variety of neuropsychiatric conditions such as AD, MS, HIV encephalitis, and traumatic brain injury involve inflammatory activation of microglia, the cerebral equivalent of the macrophage. Neutrophil infiltration is seen with ischemic stroke, cerebral infection, and other conditions. Astrocytes, also activated in neuropsychiatric disease, show many neuroimmunologic similarities to macrophages and microglia, and express inflammatory cytokines. We hypothesized that microglia and astrocytes would express MIP-1α and that this expression would be regulated by inflammatory stimuli. Expression of MIP-1α mRNA by an immortalized microglial cell line (BV-2), and primary mouse cortical astrocyte cultures was examined with RT-PCR. We found that in both microglia and astrocytes MIP-1α mRNA was strongly induced by 10 μg per ml medium of LPS and 50 ng per ml of medium of the phorbol ester PMA. The proinflammatory cytokine TNF-α and IL-1β also increased MIP-1α expression. MIP-1α mRNA was reduced by dBcAMP (0.25mM), and the LPS-induced increase in MIP-1α could be partially blocked with dBcAMP. LPS induction of MIP-1α was also blocked by PGE_1. These findings indicate that MIP-1α is likely to be expressed by microglia and astrocytes *in vivo* and may modulate cerebral inflammation in neuropsychiatric disease. Manipulation of glial second messenger systems alters MIP-1α expression, and hence the potential exists for pharmacologic control (Murphy et al., 1995b).

In order to further understand the mechanism of astrogliosis after a scratch wound of cultured rat astrocytes, we quantitated the changes in gene expression of *c-fos, c-jun*, heat shock protein 70 (HSP-70), vimentin, GFAP, actin, IL-1, IL-6, TNFα , and HPRT (a housekeeping gene) by the RT-PCR method in primary, secondary, and injured cultures. Two separate time course experiments, with triplicate cultures for each time point were carried out for the primary, secondary, and injured cultures. In primary culture from initial plating to 14 days, *c-fos* and HSP-70 mRNAs remained at low levels while actin, vimentin, and GFAP mRNAs increased (Fig. 2). In secondary cultures, *c-fos* and HSP-70 remained at low levels while actin decreased and vimentin and GFAP mRNAs remained at high levels (Fig. 3). Gene expression in the scratched cultures were followed from 0 time to 6 days. Within 60 minutes, *c-fos* mRNA increased by 40 fold and HSP-70 mRNA increased by 4 fold. Both returned to low levels by 12 hours following the injury. *c-Jun*, and IL-6 mRNAs were not detected in any of the cultures examined (Fig. 4). TNFα was detected following the scratch wound (Eng et al., 1994).

In this initial study with quantitative RT-PCR in the scratch wound, *c-fos* and HSP-70 were the only genes tested that showed rapid and significant increases following mechanical injury. This early induction of *c-fos* mRNA is consistent with the findings from previous studies in brain trauma and ischemia in rat brain and astrocyte cultures (Dragunow et al., 1990; Phillips and Belardo, 1990; Blumenfeld et al., 1992; Yu et al, 1994). Activation of the inducible HSP-70 gene by mechanical injury is similar to its induction in astrocyte cultures heated at 45°C for 20 minutes (Dwyer et al., 1991) or experimental allergic encephalomyelitis (Aquino et al., 1993). The gradual increase in GFAP mRNA with time following injury is consistent with the increase of GFAP found in this model (Yu et al., 1993a).

Primary response genes share common promoter elements such as the serum response element (SRE), TPA response element (TRE), and cAMP response element (CRE). The *c-fos* SRE, located at –300 basepairs (bp) from the transcription start site, mediates induction by many growth factors (reviewed by Treisman, 1990) whereas agents that elevate cAMP or calcium levels generally target the CRE at –60 bp (Sheng et al., 1988). No physiological role for the *c-fos* TRE at –295 has to our knowledge has been assigned. Other sequences important for transcriptional regulation of *c-fos* include a re-

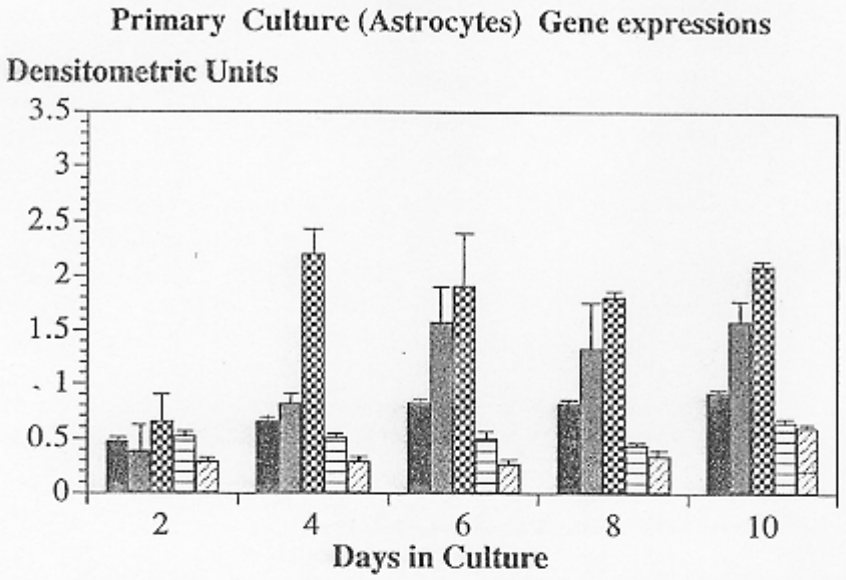

Fig. 2. Densitometric analysis of GFAP, vimentin, *c-fos*, HSP-70, and actin mRNAs in primary astrocyte cultures for ten days. The results represent an average of triplicate cultures at each time point and the average of two different experiments.

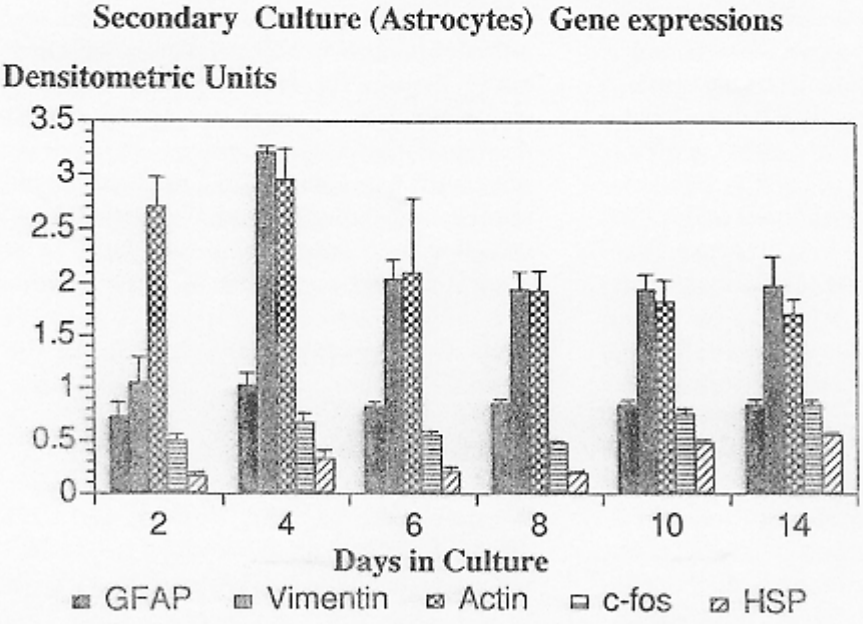

Fig. 3. Densitometric analysis of GFAP, vimentin, *c-fos*, HSP-70, and actin of mRNAs in secondary astrocyte cultures for fourteen days. The results represent an average of triplicate cultures at each time point and the average of two different experiments.

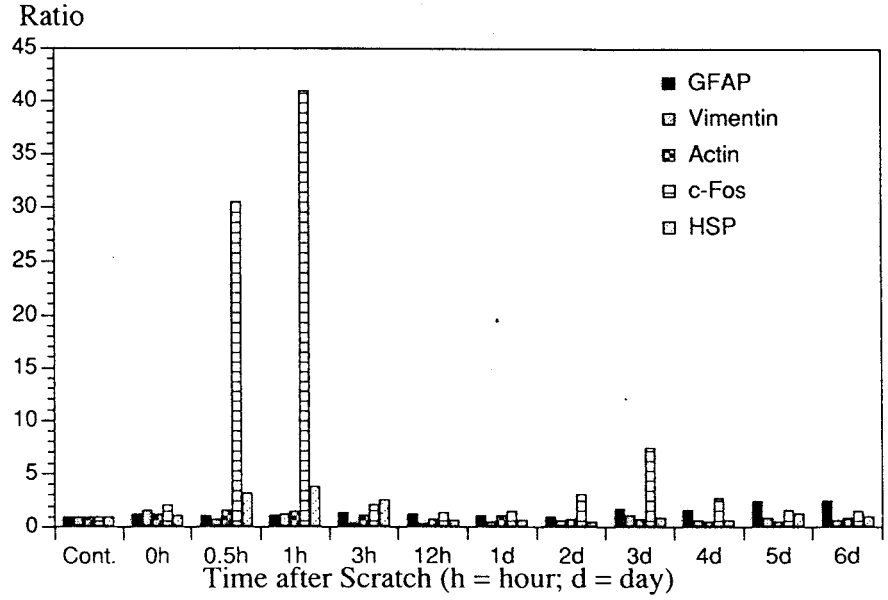

Fig. 4. Ratios of densitometric units for GFAP, vimentin, *c-fos*, HSP-70, and actin mRNAs in scratch injured secondary astrocyte cultures divided by those in untreated secondary astrocyte cultures.

gion at –345 bp, which participates in platelet-derived growth factor induction of *c-fos* and is bound by as yet an uncharacterized protein (Hayes et al., 1987), and an element at –276 bp termed SRE-2 (Visvader et al., 1988), which is important for NGF induction and is bound by factors distinct from those that bind to the SRE (Visvader et al., 1988). The retinoblastoma protein has been shown to repress *c-fos* expression through an element located at –90 bp within the *c-fos* promoter (Robbins et al., 1990). It is not surprising that *c-fos* can be induced by virtually any type of stimulus, including phorbol esters, neurotransmitters, or injury. *c-fos* activation in our mechanical injury model is similar to induction by centrifugation of cultured cells (Curran, 1988). *c-fos* induction can be due to mitogenic activation or to differentiation. Whether the *c-fos* or HSP-70 up-regulation is a response of all astrocytes or only the damaged or dividing cells is not known, but is presently under investigation.

In summary, the study of scratch wound astrocyte cultures will determine which of the many factors reported in reactive astrocytes are induced or up-regulated in the absence of damaged neurons, oligodendrocytes and microglia. With this information, one may be able to identify the cellular source of each factor and whether each response is beneficial or detrimental to CNS regeneration. This information may allow one to design specific treatments to promote regeneration and function in the injured CNS.

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