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Thrombospondin and tumor necrosis factor

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Progress in two main projects in my laboratory are discussed: (i) the study of the extracellular matrix/cell surface molecule thrombospondin (TSP); and (ii) the characterization of primary or immediate early response genes induced by tumor necrosis factor-α (TNF).

Thrombospondin

Thrombospondin (TSP) is a platelet and cell derived homotrimeric glycoprotein of molecular weight 450,000 daltons. TSP is released from platelet α-granules upon activation and plays an essential role in the secretion dependent phase of platelet aggregation. In addition, TSP is synthesized and secreted by a variety of cells in culture and is a component of the extracellular matrix. TSP binds a variety of ligands including heparin, collagen, fibronectin, fibrinogen, plasminogen and plasminogen activator [reviewed in 1]. These interactions are mediated by various protease resistant regions or domains that compose the TSP molecule. One such domain is the amino terminal heparin binding domain that contains a high affinity site for heparin. This domain had been identified by subjecting proteolytic fragments of TSP to heparin Sepharose chromatography. The isolated heparin binding domain is a monomeric peptide of 25,000 dalton molecular weight. Since the N-terminal amino acid sequence of the heparin binding domain is identical to that of the intact TSP molecule, the heparin binding domain must reside at the very N-terminus of TSP [2]. The amino acid sequence of the heparin binding domain as predicted from the nucleotide sequence of TSP cDNA reveals it to be hydrophobic in character and to contain two clusters of basic amino acids that contribute to the binding of anionic heparin [3]. A number of findings suggest that the heparin binding domain is of major physiological relevance. Heparin, presumably by binding to the heparin binding domain, inhibits the incorporation of radiolabeled TSP into isolated cell matrices. In addition, by the same mechanism heparin inhibits the incorporation of newly synthesized TSP onto the cell surface of aortic smooth muscle cells. Recent evidence suggests that the cell surface receptor for TSP is a heparan sulfate proteoglycan and heparin by competing for the binding of TSP to this receptor is capable of inhibiting cell surface association [4, 5]. A variety of evidence which is outlined below suggests that TSP plays an important role in the growth response of aortic smooth muscle cells.

When quiescent aortic smooth muscle cells are treated with a mitogen, platelet derived growth factor (PDGF), TSP levels are induced rapidly, (that is, within 15 min) and in a dose dependent manner. Importantly, the induction of TSP message parallels PDGF mediated mitogenesis. Further, in the presence of the protein synthesis inhibitor cycloheximide, PDGF superinduces the TSP transcript [6]. As a whole, these data suggest that mRNA levels for TSP are regulated by PDGF in a manner similar to that of c-*myc*, c-*fos* and other growth regulatory gene products. This rapid induction of TSP message together with its superinducibility in the combined presence of PDGF and cycloheximide suggests that TSP may be a member of the competence gene family that mediates the mitogenic response of cells to PDGF [6]. Once synthesized and secreted, TSP binds to the smooth muscle cell surface and extracellular matrix where it plays a growth facilitative role. For example, TSP and epidermal growth factor (EGF) act synergistically to stimulate DNA synthesis by smooth muscle cells. The 30-hour nuclear labeling index increases from a mean of 7% in quiescent cells to 20% for EGF treated smooth muscle cells. However, TSP and EGF act synergistically, stimulating DNA synthesis to give a labeling index of 47%. This facilitative effect of TSP on EGF mediated smooth muscle cell proliferation is inhibited by heparin. Heparin, a known inhibitor of aortic smooth muscle cell growth and migration, inhibits the incorporation of TSP into the aortic smooth muscle cell surface/matrix compartment. This is a dose dependent phenomenon with a maximal effect seen at a heparin concentration of 1 μg/ml. Thus, it is conceivable that the antiproliferative effect of heparin is mediated through its inhibition of incorporation of TSP into the extracellular matrix. Support for such a hypothesis comes from studies showing that either polyclonal or monoclonal antibodies against TSP inhibit aortic smooth muscle cell proliferation and migration [7]. Aortic smooth muscle cells treated with an anti-TSP monoclonal antibody results in a greater than 60% reduction in cell number by the eighth day. These cells are morphologically identical to control cells and when analyzed by flow cytofluorometry are found to be arrested in the G1 phase of the cell cycle [7]. The anti-TSP monoclonal antibody-like heparin abolishes cell surface expression of TSP as demonstrated by immunofluorescence and metabolic labeling experiments [7]. These studies suggest that cell surface associated TSP is functionally essential for smooth cell proliferation and that this requirement is located in the G1 phase of the cell cycle. Agents that perturb the interaction of TSP with the smooth muscle cell surface such as heparin or anti-TSP antibodies inhibit smooth muscle cell proliferation [7]. Recently, it has been shown that TSP deposition occurs in vivo following rat carotid artery injury. One hour following balloon catheter injury, prominent cell associated

TSP immunostaining is evident in the media. In this rat model, intimal proliferation is evident five days after balloon injury. TSP staining is especially profound in the neointima and media compared to non-operated controls. This in vivo data supports the hypothesis that TSP expression is an early response to injury and is localized predominantly to areas of smooth muscle cell proliferation.

The mechanism by which TSP promotes cell migration is likely related to its ability to act as a focus for protease generation. TSP has previously been shown in in vitro assays to bind both plasminogen and plasminogen activator resulting in a 40-fold increase in the efficiency of plasmin generation. Furthermore, the plasmin generated remains bound to TSP and is protected from inactivation by plasmin inhibitors [8]. More recently, specific complex formation of TSP with urokinase has been demonstrated and these complexes as well are protected from inhibition by plasminogen activator inhibitor. These workers also demonstrated co-distribution of TSP and urokinase in normal and malignant breast tissue. Thus, TSP may play an important role in the extracellular matrix via its ability to facilitate matrix degradation and remodeling that accompanies the proliferation and migration of mesenchymal and tumor cells [9]. We have explored further the potential role of TSP in tumor cell invasiveness by utilizing a genetic approach. We transfected a highly invasive squamous carcinoma cell line that produced large amounts of TSP with a TSP cDNA antisense expression vector. Three unique transfected clones were characterized, all of which expressed the transfected antisense transcript to varying degrees as verified by a ribonuclease protection assay. Most importantly, these clones showed corresponding reduction in TSP protein and interestingly a corresponding decrease in invasive potential, further emphasizing a role for TSP in cell migration [10]. Given the facilitative effects of TSP on cell growth and migration we have embarked upon examining what role TSP may play in embryogenesis. As assessed by immunocytochemistry TSP is densely deposited in the developing nervous system in regions of cell migration [11, 12]. For example, during histogenesis of the cerebellar cortex, TSP is found deposited around granule cells as they migrate through the molecular layer. When anti-TSP antibodies are added to explant cultures of cerebellar cortex during active granule cell migration, a dose-dependent inhibition of migration is observed. In control cultures, granule cells migrate into the internal granule cell layer, while granule cells exposed to anti-TSP antibodies are arrested within the external granule cell layer. These results suggest that TSP plays an important role in the histogenesis of the cerebellar cortex by influencing cell migratory events [11].

Until recently, all the myriad activities associated with TSP ranging from platelet aggregation to neurite outgrowth have been assigned to a single molecule with little evidence for alternate forms generated either by alternative splicing or as members of a gene family. However, the existence of a gene family has received strong support from our recent finding of a second expressed TSP gene whose gene product has been designated TSP2 [13].

Comparison of TSP1 and TSP2 amino acid sequences reveals a gradient of sequence conservation, with the amino terminus which encodes the heparin binding domain being most distinct and the carboxy terminus most similar [13]. Differences in the heparin binding domain may cause altered affinities for cell surface and matrix proteoglycans with resultant alteration in the distribution of the two TSPs. The discovery of a second TSP raises a number of questions including: (i) is its synthesis regulated by growth factors; (ii) is it trimeric and if so, does it form homo or hetero trimers; (iii) does it mediate cell attachment, platelet aggregation, or neurite outgrowth; (iv) what is the nature of the cell surface receptor(s) which binds it and; (v) are there more than just two TSPs?

Tumor necrosis factor

Tumor necrosis factor-α (TNF) is a cytokine that appears to have a key role in orchestrating the inflammatory response. TNF, by affecting many target cell types, is capable of inducing a wide spectrum of pro-inflammatory activities and has been implicated in mediating a variety of disease states [reviewed in 14]. An important target cell type is the vascular endothelium. Its strategic location at the interface of the bloodstream and the tissues makes it of particular interest in inflammatory processes. Far from being a passive target in inflammation as had been previously assumed, it is now known to be an active participant in processes relevant to inflammation, including those processes induced by TNF and other cytokines. For example, TNF causes an orchestrated change in the endothelial coagulation phenotype from anti- to pro-coagulant. It induces the expression of a tissue factor-like molecule and a plasminogen activator inhibitor, while decreasing expression of plasminogen activator and suppressing the protein C pathway. TNF also induces or increases the expression of adhesive molecules such as endothelial-leukocyte adhesion molecule-1 and intercellular adhesion molecule-1 on the endothelial surface, events which promote interaction of circulating leukocytes with the endothelium and that can be expected to be pro-inflammatory.

The complex and pleiotropic action of TNF on endothelial cells suggests that this cytokine may ultimately evoke the expression of a wide variety of cellular genes. Is seems likely that such genes may be members of two broad classes. The first class consists of genes which do not depend on cellular protein syntheses. As such, these would be akin to the "primary response" or "immediate early" genes induced in quiescent cells by the addition of growth factors [15]. The second class of genes requires protein synthesis for its expression. Since the former class of genes represents the initial cellular response to TNF, its characterization might be expected to provide valuable insights into the mechanism of action of TNF. Indeed, the characterization of serum inducible primary response genes has led to a better understanding of the mechanism by which growth factors exert their mitogenic effect. From these studies it is evident that primary response genes fall into two categories. The first category encodes transcriptional factors including c-*fos*, c-*myc*, c-*junA*, c-*junB* and zinc finger containing nuclear proteins [16]. In the second category are paracrine factors which influence other cells and include the secreted proteins JE and KC which have significant homology to T cell cytokines [16 and references therein]. It should be noted that both categories of primary response genes encode *cascade initiating* proteins that are capable of influencing a large number of cellular events. It is obvious, for example, that induction of a transcriptional factor may activate or repress the transcription of a large number of genes.

In contrast to growth stimulatory products such as serum, EGF and PDGF, some cytokines are growth inhibitory. In the case of TNF, the addition of even small amounts to cultured endothelial cells in vitro results in a rapid but reversible loss of

proliferative activity [17]. At present, it is unclear what is the nature of primary response genes in cells stimulated with growth inhibitors. Do the primary response genes in such an instance encode anti-oncogenes, that like the retinoblastoma gene product serve to block cell cycle progression?

In an attempt to answer these questions, and to begin to understand the mechanism of TNF action, we undertook the cloning and characterization of *primary* response genes induced by this growth inhibitory cytokine in endothelial cells.

As it was likely, given its pleiotropic biological effects, that TNF directly and indirectly influenced the transcription of a very large number of genes, we attempted to limit our analysis to the cloning of *primary* response genes. Such genes characteristically undergo *rapid* and *profound* induction of transcription and are not dependent on intermediary protein synthesis. In this section we will discuss: a) The strategy used to clone *primary* response genes; b) Induction of the nuclear protooncogene c-*jun* by TNF; and c) TNF mediated induction of other primary response genes (including three novel ones).

Cloning of TNF-induced primary response genes by differential hybridization

To restrict the analysis to primary response genes, so that gene induction was a *direct* consequence of TNF stimulation, the induced cDNA library was constructed from endothelial cells treated with both TNF and cycloheximide (CHX). Using 10 μg of poly $(A)^+$ RNA from TNF/CHX treated cells, a cDNA library was constructed in the vector γgtll. Ligation and packaging of the cDNA resulted in a library of 5×10^6 independent recombinants. Approximately 30,000 plaques of this cDNA library were screened by differential hybridization with single stranded cDNA probes complementary to mRNA from both untreated and TNF/CHX treated cells. Briefly, the differential plaque hybridization was performed by plating phage at low density in 150 cm^2 dishes (2 to 5 plaques/cm^2). A duplicate set of nitrocellulose filters was taken from each dish and hybridized with high specific activity ^{32}P labelled cDNA probes (2 to 8×10^9 cpm/μg) synthesized by the reverse transcriptase reaction from poly $(A)^+$ RNA. Plaques preferentially hybridizing with cDNA probe from TNF/CHX-stimulated endothelial cells were rescreened and finally plaque purified. Differential screening in this manner led to the identification of 200 cDNAs that were preferentially expressed in TNF-stimulated endothelial cells [18]. The differentially hybridizing cDNAs were screened with probes for genes known to be induced by TNF. These included plasminogen activator, c-*fos*, c-*myc*, HLA class 1, CSF-1, GM-CSF, intercellular adhesion molecule-1, and tissue factor [14 and references therein]. Only one hybridizing cDNA (to plasminogen activator inhibitor) was detected, possibly because the transcription of these other induced genes was dependent on protein synthesis. Based on the intensity of the differential signal, 40 of the 200 cDNAs were selected for further analysis by cross hybridization. Cross hybridization studies were hampered by the presence of a repetitive sequence in eight of the 40 cDNAs. The remaining cDNAs arose from seven unique genes which were studied further by DNA sequencing, Northern blotting and nuclear run on analysis [18].

Induction of the nuclear protooncogene c-jun *by TNF*

One of the TNF induced differentially hybridizing cDNAs was found to encode AP-1/c-*jun* on sequence analysis [17]. The AP-1/c-*jun* product is a nuclear protein that forms heterodimeric complexes with the c-*fos* gene product to stimulate transcription of genes which contain AP-1/c-*jun* response elements. AP-1/c-*jun* bears similarities to other so-called "immediate early" or "competence" genes such as c-*fos* and c-*myc* in that its expression is suppressed in cells that have been allowed to enter the G_0 or quiescent state. However, mitotic stimulation with serum, purified growth factors or, in some cases, phorbol esters results in a rapid transcriptional activation of these genes that is independent of *de novo* protein synthesis. Current concepts of the mitogenic response hold that the coordinate orchestration of the expression of these genes is necessary to permit reentry into the G phase of the cell cycle. Indeed, in fibroblasts where TNF is mitogenic there is concomitant induction of both c-*fos* and AP-1/c-*jun* [17, and references therein]. In contrast, we found that in endothelial cells the antiproliferative activity of TNF is accompanied by the transient transcriptional activation of the AP-1 gene. More importantly, there is *no* concomitant expression of c-*fos* or c-*myc* transcripts [17]. Together, these results argue that stimulatory and inhibitory cytokines induce distinct subsets of immediate early transcripts which may, in certain cases, contain common members such as AP-1/c-*jun*. The availability of multiple cDNA clones representing other "immediate early" transcripts in TNF-treated human umbilical vein endothelial (HUVE) cells will now enable us to explore more fully the molecular basis of this response and to document more accurately the overlap between mitogenic and antimitogenic stimuli postulated here. In any event, our results argue that the AP-1/c-*jun* and c-*fos* genes are controlled by separate inductive signals. Recent evidence indicating a negative feedback loop of c-*fos* protein on the c-*fos* gene and a positive feedback loop of AP-1/c-*jun* protein on its cognate gene supports this notion.

Cloning of other TNF-induced primary response genes in HUVE cells

The other six differentially hybridizing cDNAs were further characterized by both DNA sequencing and Northern blot analysis. This revealed that two of the induced cDNAs were identical to recently described, cytokine-induced endothelial cell gene products: neutrophil chemotactic factor (NCF), and endothelial leukocyte adhesion molecule-1 (ELAM-1). One of the induced cDNAs was identical to monocyte chemotactic factor (MCF) which was not a known endothelial cell product, but has been recently identified and characterized from a human glioma cell line, a human promyelocytic cell line HL-60, and a human myelomonocytic cell line [18 and references therein]. The remaining three cDNAs (A20, B61, B94) are not identical to any known gene product and represent novel TNF induced genes. A short discussion of them follows.

A20. Sequence analysis of a 4.2 kb cDNA revealed that A20 contains a remarkable number of zinc finger motifs (seven) towards its carboxy terminus [19]. Zinc finger motifs were originally identified as a DNA binding structure in TFIIIA, an RNA polymerase III transcription factor [19, and references therein]. However, since then other zinc fingers have been described in DNA binding proteins that are involved in RNA polymerase II-mediated transcription. They can be classified into two classes: (i) C_2H_2 category, which is exemplified by TFIIIA and consists of proteins containing a highly conserved

basic structural unit composed of pairs of cysteines and histidines separated by a loop of twelve amino acids. The cysteine and histidine residues stabilize the domain by tetrahedrally coordinating a Zn^{2+} ion. (ii) Cx category is exemplified by the DNA binding domain of steroid and thyroid hormone receptors [19, and references therein]. This motif uses a variable number of conserved cysteines for metal chelation. A20, like the steroid receptors, contains four conserved cysteines $C\text{-}X_4\text{-}C\text{-}X_{10\text{-}13}\text{-}C\text{-}X_{\text{-}2}\text{-}C$, where C represents the conserved cysteine and X indicates intervening amino acid residues. It is important to note that other than the conserved cysteines, the sequence of A20 is not identical to members of the human thyroid/steroid receptor family. Further, since discovery of zinc finger motifs is considered diagnostic of transcription factors, A20 represents an excellent candidate for a novel cytokine activatable transcriptional regulatory molecule. In keeping with its proposed DNA binding function A20 contains a fair enrichment in basic residues within its finger domains [19].

B61. The 1.55 kb B61 mRNA is induced within 15 minutes in human umbilical vein endothelial (HUVE) cells after TNF stimulation. This induction is largely a result of increased transcriptional rate as assessed by nuclear run-on experiments. B61 message is similarly induced by IL-1 and lipopolysaccharide, but not by gamma interferon or by endothelial cell growth factor. Additionally, B61 mRNA expression is induced by TNF and IL-1β in both keratinocytes and IMR-90 fibroblasts in culture. Sequence analysis of cDNA clones encoding B61 reveal that its protein product has no significant homology to previously described proteins. B61 is primarily a hydrophilic molecule and contains a hydrophobic N-terminus, which is consistent with the secreted nature of the protein. The mature form of the predicted protein consists of 187 amino acid residues and has a predicted molecular weight of 22,000 daltons. Immunoprecipitation of metabolically-labeled HUVE cell preparations indicates that B61 is a 25-kilodalton secreted protein which is markedly induced by TNF [20, and references therein].

Since all known TNF induced and secreted proteins derived from endothelium are paracrine factors such as IL-1, IL-6, IL-8, MCP-1, M-CSF, G-CSF, GM-CSF, and PDGF [14], we believe that B61 will fall into this category.

B94. B94 is a 4.2 kb transcript that is induced rapidly, within an hour of exposure to TNF. Furthermore, in the concomitant presence of cycloheximide, the B94 transcript is superinduced which is consistent with it being a primary response gene. Other proinflammatory stimuli like interleukin-1β and lipopolysaccharide are also capable of inducing B94 transcript. Nuclear run-on studies show that the induction of B94 occurs largely at the transcriptional level. The induction of B94 protein, as assessed by immunoprecipitation, parallels induction of the transcript both in terms of rapidity and magnitude of induction. The protein sequence of B94 is not homologous to that of any known protein. However, there are segments that have identity or close similarity to conserved sequences found in protein kinase catalytic domains, raising the intriguing possibility that B94 may be a kinase that is transcriptionally activated.

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