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Induction of human tenascin (neuronectin) by growth factors and cytokines: cell type-specific signals and signalling pathways

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SUMMARY

The extracellular matrix protein tenascin (TN) is expressed with precise temporo-spatial patterns during embryonic and fetal development and is induced in healing wounds, inflammatory lesions and solid tumors. These tissue patterns suggest that TN synthesis may be modulated by soluble factors present in developing tissues or released from injured, inflammatory or neoplastic cells. To characterize the extrinsic control of human TN we examined the effects of several signalling molecules on cultured neural, melanocytic and fibroblastic cells. Results obtained with αTN antibodies in enzyme-linked immunosorbent and immunoprecipitation assays indicate that TN expression is tightly regulated in a cell type-specific manner: (1) Primitive neuroectodermal tumor (PNET) cells grown in chemically defined, serum-free media show up to >100-fold TN induction in response to fibroblast growth factors (aFGF, bFGF, K-FGF) and phorbol ester, independent of changes in cell proliferation or total protein synthesis; no induction is seen in PNET cultures stimulated with serum or other growth and differentiation factors. (2) Normal melanocytes, which require FGF and phorbol ester for survival in vitro, fail to express TN; however, they produce TN following oncogenic transformation. (3) Fibroblasts derived from disparate tissues differ up to 100-fold in basal TN production; for example, fetal lung fibroblasts are TN^{high}, but conjunctival fibroblasts derived from the same donors and fetal leptomeningeal cells are TN^{low}. (4) TN^{low} fibroblasts treated with interleukin-1, tumor necrosis factor-α, and interleukin-4 show up to >100-fold increased TN secretion and TN incorporation into their extracellular matrix. Transforming growth factor-β, which acts as an inducer of fibronectin, collagen, and integrin-type matrix receptors, has variable effects on fibroblast TN, ranging from increased deposition in the extracellular matrix of fetal conjunctival fibroblasts to reduced secretion in newborn foreskin fibroblasts. In contrast, FGFs (which are potent fibroblast mitogens), phorbol ester, bone morphogenetic proteins, and several other factors tested produced no discernible effects on fibroblast TN expression. These findings suggest that discrete sets of extrinsic signals modify TN expression in specific cell types, with the effects of a given ligand/receptor system determined by cell type-specific signalling pathways that may be linked to unique cis-regulatory elements of the TN gene. As a result, a limited set of regulatory peptides may produce highly diversified TN distribution patterns in developing and lesional tissues.

Key words: tenascin, neuronectin, cytotactin, hexabrachion, extracellular matrix, growth factor, cytokine, phorbol ester, interleukin

INTRODUCTION

The extracellular matrix (ECM) contributes to cell migration and pattern formation during development and is likely involved in wound healing, inflammation, cancer and other diseases (Edelman and Crossin, 1991; Liotta and Stetler-Stevenson, 1991). One of the ECM proteins that has been studied extensively in developing and lesional tissues is tenascin (TN) (reviewed by Erickson, 1993), a disulfide-bonded multimer of M_r 180,000-250,000 subunits that assembles into characteristic six-armed structures, so-called hexabrachions (HxB) (Erickson and Iglesias, 1984). Biochemical and molecular genetic studies have shown that TN polypeptides: (i) are encoded by a single gene; (ii) comprise multiple domains with sequence similarity to epidermal growth factor, fibronectin type III repeats, and a fibrinogen-like domain; and (iii) show isoform-diversity due to alternative mRNA splicing. Structurally similar, although not identical, TN proteins have been identified in a wide range of species, including chickens, rats, mice, humans, cows and pigs, and are variously referred to as TN, HxB, cytotactin (CT), myotendinous antigen, $J1_{220/200}$, glial-mesenchymal extracellular matrix protein (GMEM), or neuronectin (NEC1) (Erickson, 1993). (In the present report, the name TN is used for these proteins in all species; however, serological reagents are referenced according to their original designations because of differences

in isoform specificity, recognition of species-specific or shared epitopes, and possible cross-reactions with unrelated proteins).

Despite the structural similarities among TN proteins in different species, their tissue distribution patterns may show some species-specificity (Rettig et al., 1992b). The distribution of human TN in normal, reactive, and neoplastic tissues has been studied extensively (Bourdon et al., 1983; Garin-Chesa et al., 1989; Lightner et al., 1989; Mackie et al., 1987, 1988; Rettig et al., 1988, 1992b) and four different patterns have been defined. A region-specific distribution is observed in the adult central nervous system, which shows rostro-caudal patterning of TN, and in the skin where TN is expressed in the dermal papillae of hair follicles and along the dermal-epidermal junction. More widespread, organ- or tissue-specific TN expression is observed in the kidney, liver, spleen, and smooth muscle. Transient TN expression during specific stages of fetal development is seen in the precartilage blastemas of developing bones. Finally, a number of tissues that normally lack TN show induction of the molecule during reactive, inflammatory, or neoplastic changes, as exemplified by the granulation tissue of healing wounds, the stromal compartment of epithelial cancers, and skin lesions such as actinic keratoses and psoriasis. These distribution patterns suggest that both tissue-specific, intrinsic factors and extrinsic factors present in embryonic tissues or released from injured, reactive, or neoplastic cells modulate TN production.

Initial evidence for TN induction by extrinsic factors came from studies with serum-starved chicken embryo fibroblasts (CEFs), which show a 4-fold increase in TN mRNA and protein levels when treated with transforming growth factor-β (TGFβ) (Pearson et al., 1988). Subsequent studies have identified additional inducers of TN in other cell types and species. For example, fibroblast growth factors (FGF) augment TN production by human neural cells (Rettig et al., 1989), tumor necrosis factor-α (TNF-α) induces TN expression in adult human skin and lung fibroblasts (Rettig and Garin-Chesa, 1989), and interleukin-1 (IL-1) increases TN production by human synovial fibroblasts (McCachren and Lightner, 1992). Among the rodent cells tested, rat vascular smooth muscle cells upregulate TN expression when treated with angiotensin II or TGFβ (Sharifi et al., 1992; Mackie et al., 1992), rat C6 glioma cells show increased expression with nerve growth factor treatment (Yavin et al., 1991), and FGF and TGFβ augment TN production in mouse Swiss 3T3 fibroblasts (Tucker et al., 1993).

Although several potential inducers of TN have now been identified in different cell types and species, the rules that determine the specificity of TN induction and the possible interactions between multiple factors have not been established. Therefore, the present study was designed to compare TN induction in human cell types of neural, melanocytic, and fibroblastic origin and to determine whether TN regulation is linked to changes in cell proliferation, malignant transformation or production of other cellular or secreted proteins.

MATERIALS AND METHODS

Cell lines and cell culture

Primitive neuroectodermal tumor (PNET) cell lines 6647, TC-149, and TC-215 (alternatively referred to as peripheral neuroepitheliomas or Ewing's sarcomas; Rettig et al., 1992a) were obtained from Dr T. J. Triche (Children's Hospital, Los Angeles). Normal melanocyte cultures and H-*ras*-transformed melanocyte strains have been described (Albino et al., 1992). GM-series fibroblasts were from the National Institute of General Medical Sciences (Camden, NJ). WI-38, Hs27 and Hs68 fibroblasts were obtained from the American Type Culture Collection (Rockville, MD), and other fibroblast cultures were established from surgical specimens. Cell lines were grown in RPMI1640 or MEM media with amino acids and antibiotics, and supplemented with 10% fetal bovine serum (FBS), 0.5% FBS, or ITS+ (6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenium, 5.35 μg/ml linoleic acid, and 1,25 mg/ml bovine serum albumin; Collaborative Research, Bedford, MA). For cell proliferation and viability assays, cells cultured in Falcon 3047 plates were trypsinized at different time points, stained with trypan blue, and counted in a phase-contrast hemacytometer (American Scientific Products).

Growth and differentiation factors

The following factors (range of concentrations) were tested: human transforming growth factor-β1 (TGFβ1, 0.1-10 ng/ml; R&D Systems, Minneapolis, MN); recombinant human interleukin-1α (IL-1α, 0.1-10 ng/ml), IL-1β (1 ng/ml), IL-4 (0.1-10 ng/ml), IL-6 (100 ng/ml), and platelet-derived growth factor (PDGF)-B chain homodimer (PDGF-BB, 5-50 ng/ml; Genzyme, Boston MA), epidermal growth factor (EGF; 10-100 ng/ml), insulin-like growth factor (IGF-1; 10-50 ng/ml), recombinant human tumor necrosis factor-α (TNFα, 0.1-100 ng/ml; Genentech, San Francisco, CA), FGFs (aFGF, bFGF, R&D Systems; K-FGF, 0.1-50 ng/ml; Dr C. Basilico, New York University), recombinant human bone morphogenetic proteins (BMP-1, BMP-2, BMP-3, 20-50 ng/ml; Genetics Institute, Cambridge MA), TPA (1-10 ng/ml), forskolin (FSK, 100-200 μM), actinomycin D (0.1 μg/ml), cycloheximide (10 μg/ml; Sigma Chemical Corp., St Louis, MO), interferon-γ (IFNγ, 100-250 units/ml; Dr G. Gastl, Sloan-Kettering Institute), heparin (Lyphomed, Rosemont IL), retinoic acid, and retinol (10^{-6}-10^{-7} M; Dr J. Buck, Cornell University, New York).

Antibodies

Monoclonal antibodies (mAbs) αNEC1a, αNEC1b, Om5, AJ2 (αVLA-β1), SR84 (αVLA-α1), J143 (αVLA-α3), and F19 (αFAP) have been described (Rettig et al., 1984, 1988, 1993). Clone W6/32 (αHLA class I) was from the American Type Culture Collection, αfibronectin (αFN, human-specific) from Calbiochem (La Jolla, CA), and rabbit αhuman TN (αTN) from Telios (La Jolla, CA). Rabbit αhuman HxB (αHxB) was raised against purified human glioma HxB (Lightner et al., 1989), and rabbit αchicken CT (αchCT) has been described (Rettig et al., 1992b).

Enzyme-linked immunosorbent assay (ELISA)

TN binds avidly to tissue culture plastic surfaces, even in the presence of high concentrations of other proteins, such as 5-10% FBS or 5% bovine serum albumin (BSA), and an ELISA method for specific and highly sensitive detection of TN in spent culture supernatants (lower detection limit <10 ng/ml) based on mAb endpoint titrations has been described (Rettig et al., 1988). For quantitation of TN over a wide range of concentrations (<10 ng/ml to >50 μg/ml), we used an antigen dilution assay (Rettig et al., 1989) in which multiple ELISA determinations for 3-fold serial dilutions of purified human TN (Lightner et al., 1989) and test samples (range 1:1 to 1:243) are used to establish endpoint dilution curves. Briefly, three overlapping TN standard curves were established using 3-fold dilutions of TN in phospate-buffered saline (PBS) or RPMI1640, ranging from 2 μg/ml to 8 ng/ml, 0.2 μg/ml to 0.8 ng/ml, and 0.02 μg/ml to 0.008 ng/ml, respectively. A 15 μl sample of each TN solution was plated into the wells of MicroWell plates (Nunc, Naperville, IL), incubated overnight at 4°C or for 2 hours at 37°C, washed, and tested with mAbs NEC1a and NEC1b (αNEC1a/b), or unrelated negative control mouse IgG1 as described (Rettig et al.,

1988). Briefly, coated MicroWell plates were incubated for 1 hour with mAbs, washed, incubated for 30 minutes with alkaline phosphatase-conjugated goat antimouse-IgG (Sigma), washed, and incubated with *p*-nitro-phenylphosphate (Sigma; 1 mg/ml in diethanolamine buffer, pH 9.7) at 37°C in a humidified chamber. Changes in absorbance at 405 nm were determined on an Artek plate reader (maximal values with saturating amounts of TN and mAbs ranged from 1.9 to 2.2), and absorbance values for αNEC1a/b were corrected for nonspecific absorbance, determined with negative control mouse IgG1 (range 0.07 to 0.15). Semilogarithmic plots of the background-corrected absorbance values as a function of TN concentration were used as standard curves for tests with culture supernatants (see below). In selected experiments, up to 10% FBS or 3% BSA was added to TN standards to assess the blocking effect of concentrations of unrelated proteins; however, no significant shift in the absorbance curves were observed.

For TN induction experiments, test cells were seeded into Falcon 3047 plates under standardized conditions of cell density and viability, and cultured for various periods of time with or without inducing agents. TN levels in the culture supernatants were determined in antigen dilution assays as follows. Spent media were diluted (serial 3-fold dilution series; 1:1 to 1:243) with PBS or RPMI1640, adsorbed to MicroWell plates as described above, and tested by ELISA with αNEC1a/b or negative control mouse IgG1. Specificity controls included tests on MicroWell plates coated with RPMI1640-10%FBS, 3% BSA in PBS, or human plasma fibronectin (20 μg/ml starting concentration). For some tests with serum-free culture supernatants, 10% FBS or 3% BSA was added to the spent media prior to making serial dilutions in order to assess blocking of TN adsorption by unrelated proteins; however, no shift in the absorbance curves was observed. Since each test sample was assayed at six different dilution steps, the resulting absorbance curve could be compared directly to the TN standard curves, and TN levels in the test samples were determined from the shift of the sample curve along the abscissa. For test samples that fell into the linear range of a given TN standard curve, TN levels could also be derived from the ratio of the respective background-corrected absorbance values.

Serologic assays

The immune adherence assay for detection of cell surface and substrate-bound antigens in MicroWell plates has been described (Rettig et al., 1987). Indirect radiobinding assays with ^{125}I-rabbit antimouse immunoglobulin for cells grown in Falcon 3047 plates were carried out as described (Rettig et al., 1993).

Immunochemical procedures

Immunoprecipitation experiments were carried out as described (Rettig et al., 1992b, 1993). Briefly, cells were metabolically labeled with a mixture of [^{35}S]methionine and [^{35}S]cysteine (Tran^{35}S-label, 50 μCi/ml; ICN, Costa Mesa, CA) or with [^{3}H]glucosamine (50 μCi/ml; New England Nuclear, Boston, MA) for 18-24 hours. Radiolabeled culture supernatants, cell extracts prepared in Nonidet P40 (NP40) lysis buffer (0.5% NP40, 150 mM NaCl, 10 mM Tris-HCl, 2 mM $MgCl_2$, pH 7.4; 2 mM phenylmethylsulfonyl fluoride; 20 units/ml aprotinin), and the cell-free ECM extracted with high-pH buffer (30 mM diethylamine, 1 mM EDTA, pH 11.5, 2 mM PMSF, 20 units/ml aprotinin) were used in separate experiments. Incorporation of Tran^{35}S-label into cellular proteins was determined following acid precipitation of detergent extracts with 10% trichloroacetic acid. Purified antigens were eluted with 2% SDS-buffer and separated on 6% SDS-gels under reducing (sample buffer with 12 mg/ml dithiothreitol) or non-reducing conditions (14 mg/ml iodoacetamide), followed by fluorography. For some assays, metabolically labeled proteins were purified with concanavalin A (ConA)-Sepharose (Pharmacia, Uppsala, Sweden).

Immunohistochemical methods

Tissues were embedded in OCT compound (Miles, Naperville, IL), snap-frozen in isopentane precooled in liquid N_2, and stored at −70°C. Then 5 μm sections were cut, mounted on gelatin-coated slides, air-dried, and fixed in cold acetone for 10 minutes. The avidin-biotin immunoperoxidase procedure was used as described (Rettig et al., 1988; Garin-Chesa et al., 1989). Sections were counterstained with Harris' hematoxylin.

RESULTS

TN expression in cultured neuroectodermal cells

Primitive neuroectodermal tumors (PNET)

PNET cell lines 6647, TC-149 and TC-215 were maintained in RPMI1640-10%FBS or chemically defined, serum-free RPMI1640-ITS+ media. Fig. 1 shows the results of solid-phase

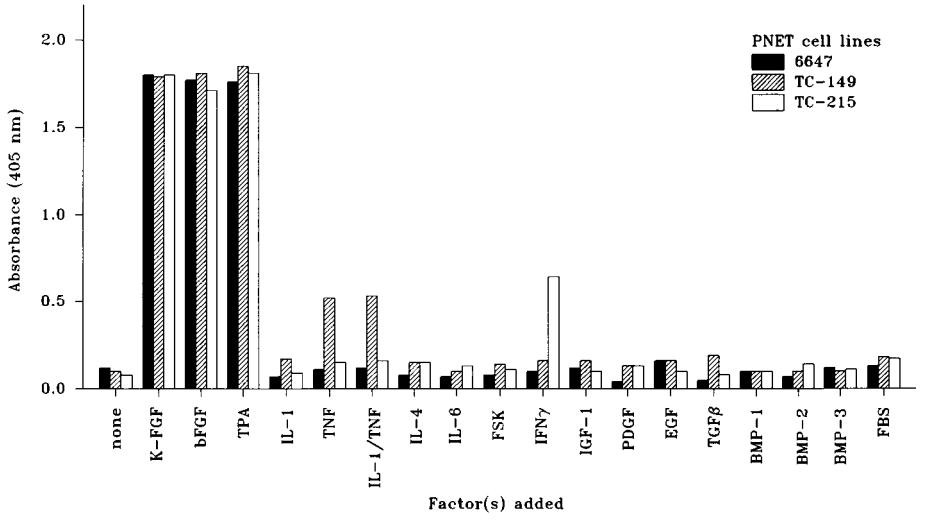

Fig. 1. Analysis of growth and differentiation factors for TN-inducing activity in PNET cell lines 6647, TC-149, and TC-215. PNET cells maintained in RPMI1640-ITS+ were seeded into Falcon 3047 plates (5×10^5 cells/well in 0.5 ml RPMI1640-ITS+) and cultured for 24 hours with the indicated factors. The 24-hour culture supernatants were diluted 1:5 with RPMI1640, adjusted to 2% FBS, adsorbed to MicroWell plates, and tested with αNEC1a/b or negative control mouse IgG1 in solid-phase ELISA. The absorbance values for αNEC1a/b are shown; background absorbance for tests with negative control mouse IgG1 was 0.08-0.11 in all tests. The factors/concentrations used were: K-FGF (25 ng/ml), bFGF (25 ng/ml), TPA (10 ng/ml), IL-1β (1 ng/ml), TNFα (50 ng/ml), IL-4 (2 ng/ml), IL-6 (100 ng/ml), forskolin (100 μM), IFNγ (200 units/ml), IGF-1 (20 ng/ml), PDGF-BB (50 ng/ml), EGF (100 ng/ml), TGFβ1 (10 ng/ml); BMP-1 (20 ng/ml), BMP-2 (20 ng/ml), BMP-3 (20 ng/ml), and 10% FBS; the full range of concentrations tested for each factor is listed in Materials and Methods.

TN-ELISA tests with supernatants of PNET cultures treated with a number of growth and differentiation factors for 24 hours. PNET cells grown in RPMI1640-ITS+ or RPMI1640-10%FBS alone produce no, or only trace amounts of, TN. However, in the presence of K-FGF, aFGF, bFGF, or TPA, they produce significant levels of TN. No TN induction was observed with most of the other factors tested, including TGFβ, PDGF, forskolin, and retinoic acid. TNFα caused a moderate increase in TN levels in one PNET line, TC-149, and IFNγ increased production by TC-215 cells.

To quantitative TN production, ELISA endpoint titration experiments were carried out with serial dilutions of 6647 and TC-149 PNET culture supernatants and compared to standard curves obtained for serial dilutions of purified TN. These tests showed that unstimulated PNET cultures produce <0.02 μg/ml TN in standard 24-hour assays (5×10^5 cells/well in Falcon 3047 plates), regardless of whether they are grown in RPMI1640-10%FBS or RPMI1640-ITS+. In the presence of bFGF (25 ng/ml) or TPA (10 ng/ml), TN levels increased to 2-5 μg/ml. When bFGF and TPA were added together, TN levels reached 4-8 μg/ml, corresponding to a >100-fold increase over unstimulated levels. TC-149 cultures treated with TNFα (50 ng/ml) and TC-215 cultures treated with IFNγ (200 units/ml) showed moderately elevated TN levels (0.07-0.2 μg/ml) after 24 hours.

PNET cells grown in serum-supplemented or serum-free media show a round, small cell morphology and grow in suspension or lightly attached to tissue culture surfaces (Rettig et al., 1992a), readily detaching with gentle shaking or following incubation with Ca^{2+}/Mg^{2+}-free PBS. None of the factors tested in this study induced significant changes in PNET morphology, substrate adhesiveness, cell numbers, viability, or total protein synthesis as measured by incorporation of Tran^{35}S-label into acid-precipitable cellular proteins.

Immunoprecipitation experiments with αNEC1a/b mAbs and αchCT antibodies revealed that the bFGF/TPA-induced TN molecules in PNET cultures comprise two major species that migrate as M_r 250,000 and 180,000 subunits on SDS-gels under reducing conditions (Fig. 2) and as disulfide-linked, high-molecular mass complexes (>M_r 1,000,000) under non-reducing conditions. In FGF-, TPA-, or TPA/FGF-treated 6647 cultures, abundant M_r 250,000 and M_r 180,000 TN species were observed, whereas TC-215 cells treated with the same factors showed predominant induction of the M_r 250,000 species.

Fig. 3 illustrates a dose-response curve for FGF-induced TN secretion in 6647 cells. All three FGFs tested were found to induce TN secretion at concentrations of 0.5 to >50 ng/ml. Similar experiments with TPA-treated 6647 cells showed TN induction at TPA concentrations of 0.02 to >20 ng/ml. In TC-149 cultures, TNFα stimulated TN production at concentrations of 0.1 to >50 ng/ml, and IFNγ increased TN production by TC-215 cells at concentrations of 20 to >200 units/ml IFNγ.

Effects of cycloheximide and actinomycin D

No increase in TN levels were obtained when PNET cells were stimulated with bFGF, TPA, TNFα, or IFNγ in the presence of actinomycin D (0.1 μg/ml) or cycloheximide (10 μg/ml), suggesting that augmented TN production requires de novo RNA and protein synthesis.

TN expression in melanocytic cells

Normal human melanocytes can be purified and grown in vitro in RPMI1640-10%FBS supplemented with bFGF and TPA (Albino et al., 1992). We examined four independently derived normal melanocyte strains (Rettig et al., 1993) maintained in RPMI1640-10%FBS with 10 ng/ml bFGF and 10 ng/ml TPA for TN production, and all four strains were nonproducers.

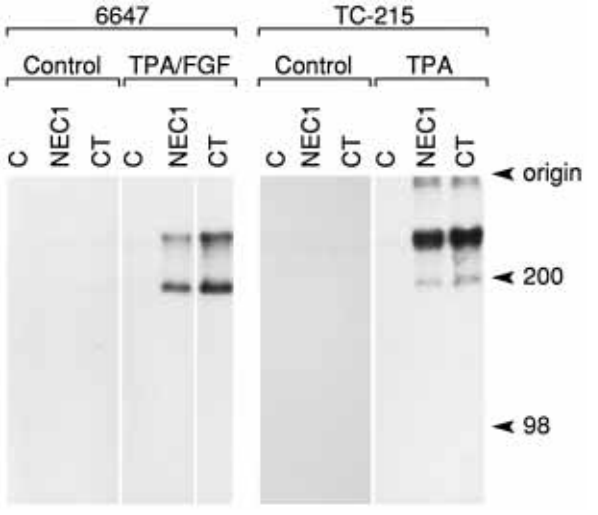

Fig. 2. Immunoprecipitation of TN from supernatants of [^{3}H]glucosamine-labeled PNET cell lines 6647 and TC-215 treated with 10 ng/ml TPA, alone or in combination with 5 ng/ml bFGF. Supernatants were tested with negative control mAb Om5 (C), αNEC1a/b, or rabbit αchCT, and immunoprecipitates were separated on a 6% SDS-gel under reducing conditions. The top of the running gel (origin) and the positions of molecular mass markers ($\times10^{-3}$) are indicated to the right.

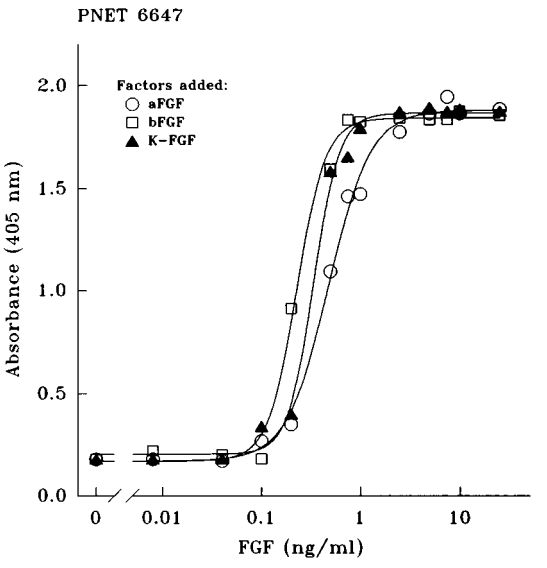

Fig. 3. Dose-response curve for FGF-induced TN secretion by PNET cell line 6647. aFGF, bFGF, or K-FGF were added to test cultures in Falcon 3047 plates (5×10^5 cells/well in 0.5 ml RPMI1640-ITS+). The 24-hour culture supernatants were adsorbed to MicroWell plates and tested by solid-phase ELISA with αNEC1a/b or negative control mouse IgG1 (background absorbance 0.08-0.11 in all assays).

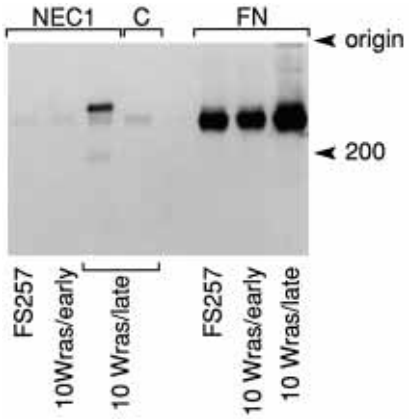

Fig. 4. Immunoprecipitation of TN and FN from Tran^{35}S-labeled normal and transformed melanocytes grown in RPMI1640-10%FBS supplemented with 5 ng/ml bFGF and 10 ng/ml TPA. The 24-hour supernatants of normal melanocytes (FS257), immortalized but nontumorigenic melanocytes (10Wras/early), and fully transformed tumorigenic melanocytes (10Wras/late) were tested with αNEC1a/b, negative control mouse IgG1 (C), or αFN, and immunoprecipitates were separated on a 6% SDS-gel under reducing conditions. The top of the running gel (origin) and the positions of molecular markers (×10^{-3}) are indicated to the right.

Thus, although FGF and TPA are essential for melanocyte survival and proliferation in vitro they are not capable of inducing TN expression in these cells. However, since some melanoma cell lines secrete TN (Rettig and Garin-Chesa, 1989), we examined whether other manipulations of normal melanocytes can activate TN expression. Using a well-characterized model of two-step transformation of normal melanocytes (Albino et al., 1992) we found that H-*ras*-transformed and immortalized but non-tumorigenic melanocytes (strain 10Wras/early) fail to produce TN but secrete large amounts of FN, similar to what is observed for primary, untransformed melanoctyes. In contrast, the fully transformed and tumorigenic melanocyte strain 10Wras/late, which was derived from the TN-nonproducing 10Wras line, secretes significant amounts of TN proteins, composed primarily of the M_r 250,000 subunit (Fig. 4). The specificity of TN induction in 10Wras/late cells is illustrated by previous studies in which the same cells were found to downregulate expression of several normal melanocyte proteins, including FAP and adenosine deaminase binding protein (Albino et al., 1992; Rettig et al., 1993).

TN expression in fibroblastic cells

Quantitative ELISA endpoint titration assays were used to assess TN production in fibroblasts from different embryonic, fetal, newborn and adult tissues (Table 1). Standardized 24-hour assays with confluent cultures (1×10^5 cells/well in 0.5 ml media, Falcon 3047 plates) revealed that some fibroblast strains secrete high levels of TN (6 to 20 μg/ml at 24 hours) whereas others produce only moderate (0.1-1 μg/ml at 24 hours) or trace amounts of TN (<0.02 μg/ml at 24 hours). Several fibroblast strains were selected for detailed analysis to determine whether organ site, developmental stage, or genetic variability among humans contribute to these >100-fold differences in basal TN production.

Fetal fibroblasts derived from distinct organ sites differ in TN production

Two sets of fibroblasts derived from different organ sites of the same fetuses were used to explore the role of specific organ site versus genetic variability in basal TN production. Fibroblast strains GM05387 (lung), GM05386A (skin), and GM05421A (conjuctiva) came from a clinically normal, 20-week-old fetus, and fibroblast strains GM05389 (lung), GM05388 (skin), and GM05420 fibroblasts (conjunctiva) came from a different 20-week-old fetus. When examined for basal TN production, both fetal lung fibroblast lines proved to secrete high levels of TN in standardized 24-hour assays (6-20 μg/ml), the skin fibroblasts produced moderate levels (0.1-0.7 μg/ml), and the conjunctival fibroblasts produced only trace amounts (<0.07 μg/ml) of TN. These ELISA results with culture supernatants were confirmed in immunoprecipitation tests with Tran^{35}S-labeled cultures, which identified the M_r 250,000 and 180,000 subunits of TN in spent media of GM05387 and GM05389 fetal lung fibroblasts but not in GM05420 or GM05421A fetal conjunctival fibroblasts (see below). The differences in TN production among GM-series fibroblasts were stably maintained during continuous culture for over 8 weeks. Three strains of leptomeningeal fibroblasts (Table 1), derived from different 20-week-old fetuses, were

Table 1. TN production in cultures of normal human fibroblasts derived from different organ sites

Fibroblast derivation			Passages	TN seretion*
Stage	Organ/tissue	Designation	tested	(μg/24 h/10^5 cells)
embryonic	lung	WI-38	23-30	0.1 - 0.35
fetal	lung**	GM05387, GM05389	6-12	3 - 10
	skin**	GM05386A, GM05388	5-8	0.05 - 0.35
	conjunctiva**	GM05420, GM05421A	6-12	<0.035
	leptomeninges**	FB2, FM3, FB20	1-12	<0.01
newborn	foreskin	Hs27, Hs68	12-25	0.1 - 0.35
adult	skin	F-HO, F-MA	2-5	0.1 - 0.35
	kidney	F-NK	4	0.015
	bone marrow	BM8, BM9	3-4	0.01
	synovium	SYN-1	1-3	0.5 - 1

*Fibroblasts in Falcon 3047 plates (1×10^5 cells/well in 0.5 ml MEM-10% FBS) were grown for 24 h, supernatants were collected, serially diluted (3-fold series: 1:1 to 1:243), adsorbed to MicroWell plates, and tested by solid-phase ELISA with αNEC1a/b and negative control mouse IgG1. Serial dilutions of purified TN were tested in parallel to establish standard curves for TN quantitation.

**One 20-week-old fetus gave rise to fibroblast strains GM05387, GM05386A, and GM05421A; a second 20-week fetus gave rise to strains GM05389, GM05388, and GM05420. Different 20-week-old fetuses gave rise to strains FB2, FM3, and FB20.

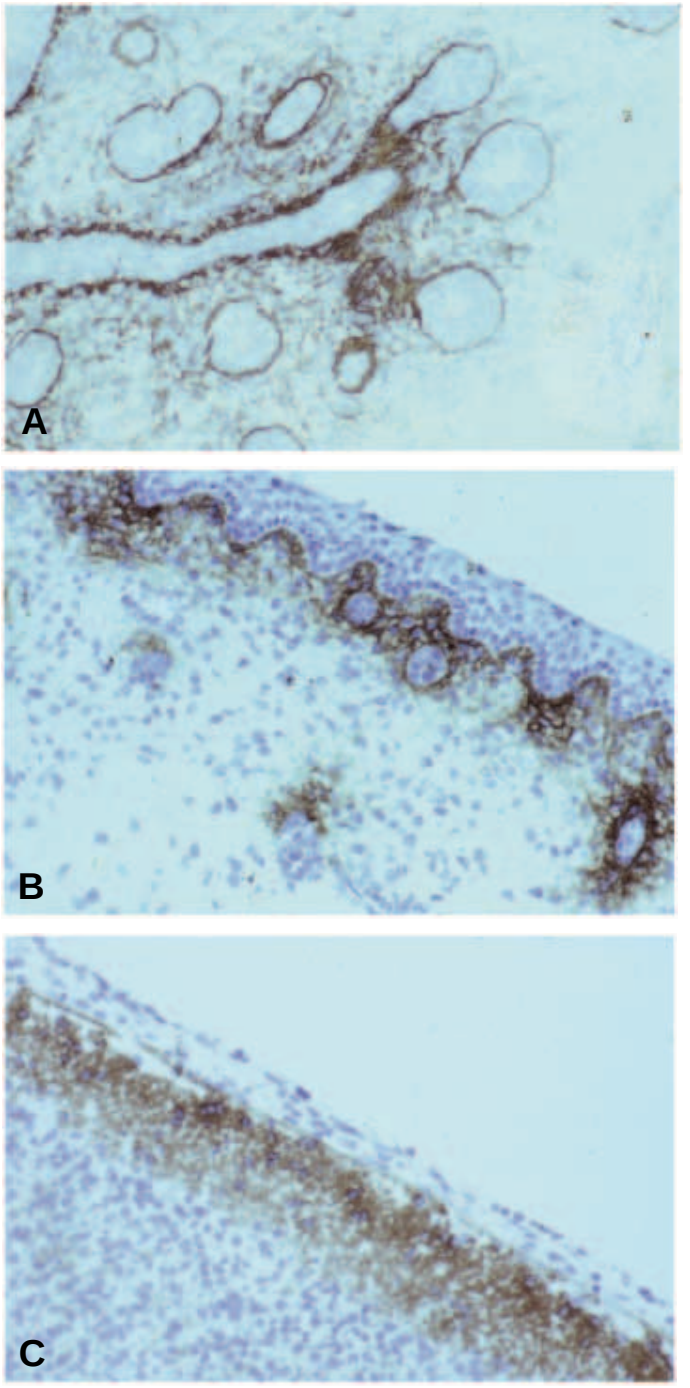

Fig. 5. Immunohistochemical detection of TN in lung (A), skin (b), and leptomeninges with adjacent brain tissue (C) of a 20-week-old fetus. Note immunostaining with a diffuse pattern along the epithelial-mesenchymal junctions in fetal lung and fetal skin, and diffuse staining in the marginal zone directly underlying the leptomeninges in the fetal brain. The loose connective tissue of the lung and dermis show no immunoreactivity. Avidin-biotin immunoperoxidase staining with mAb NEC1b and hematoxylin counterstaining. Negative control experiments with unrelated mouse IgG1 did not produce any staining.

tested over a 4 month period (passages 1 to 12) and were found to produce no or only trace levels of TN (<0.02 μg/ml).

In control immunoprecipitation and serologic assays, no differences were observed between GM-series fibroblasts with regard to FN production or expression of several fibroblast cell surface molecules, including FAP, HLA class I antigens, and β1-integrins.

TN expression in fetal tissues

Immunohistochemical analysis of selected tissues obtained from a 20-week-old fetus was carried out to determine whether the differences in TN production among cultured fibroblast strains simply reproduce differences between the in vivo TN levels of the corresponding tissues and developmental stages. However, our results indicate that all three fetal tissues contain moderate amounts of TN. Fig. 5A,B shows that in the fetal lung and fetal skin TN is present predominantly along epithelial-mesenchymal junctions. In the fetal brain, TN is seen in the marginal zone, directly adjacent to the leptomeningeal cell layer (Fig. 5C).

Peptide mediators stimulate TN production in TN^{low} GM-series fibroblasts

GM05387 and GM05389 fetal lung fibroblasts produce significant levels of TN, regardless of whether they are grown in MEM-10%FBS, MEM-0.5%FBS, or MEM-ITS+. In contrast, the fetal conjunctival fibroblast strains GM05420 and GM05421A, tested in the same media and at the same cell density and viability, produce only trace amounts of TN. Therefore we used the latter cells as targets in our search for TN-inducing factors. ELISA tests with 24-hour supernatants of standardized GM05420 and GM05421A cultures grown in MEM-10%FBS supplemented with various growth and differentiation factors (Fig. 6) showed that neither FGFs nor TPA induce TN in these cells. Instead, IL-1α, IL-1β, TNFα, and IL-4 increase their TN production. This effect was most pronounced when IL-1α (or IL-1β), TNFα, and IL-4 were added together, resulting in peak TN levels of 6 to 7 μg/ml at 24 hours. Neither TGFβ nor any of the other factors tested increased TN levels in the conjunctival fibroblast culture supernatants.

Immunoprecipitation experiments with supernatants of Tran^{35}S-labeled GM05421A cultures confirmed the increase in TN secretion in response to IL-1, TNFα, and IL-4 (Fig. 7A). Furthermore, when stimulated cultures where washed with PBS to remove soluble proteins and sequentially extracted with NP40 buffer to obtain cellular proteins (not shown), and with high-pH buffer (Fig. 8) to obtain the cell-free ECM, parallel increases in TN levels were seen in the IL-1/TNFα/IL-4-treated cultures. TGFβ-treated cultures showed some increase in TN levels in the NP40 cell extracts and high-pH ECM extracts of the same cultures. Why this increase in TN production is seen in cell extracts and the cell-free ECM but not in the culture supernatants is not presently known but may involve asymmetric secretion of TN on the basal side of TGFβ-treated cells.

When IL-1/TNFα-treated GM05421A cultures were followed for consecutive 24-hour periods, with complete changes of culture media and replenishment of factors after each period, a sustained increase in TN expression was observed for the entire 4-day test period.

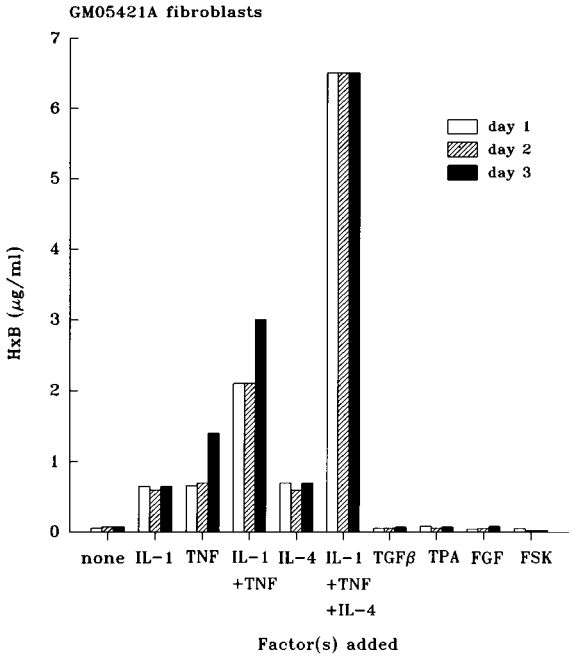

Fig. 6. ELISA analysis of TN induction (HxB) in GM05421A fetal conjunctival fibroblasts. Fibroblasts from stock cultures (passage 11) were seeded into Falcon 3047 plates (1×10^5 cells/well in 0.5 ml MEM-10%FBS) and cultured for three consecutive 24-hour periods with the indicated factors: IL-1β (1 ng/ml), TNFα (50 ng/ml), IL-4 (2 ng/ml), TGFβ1 (2 ng/ml), TPA (10 ng/ml), bFGF (25 ng/ml), or FSK (100 μM). After each 24-hour period, supernatants were collected for antigen dilution ELISA tests and TN quantitation, and cultures were refed with fresh media and the indicated factors.

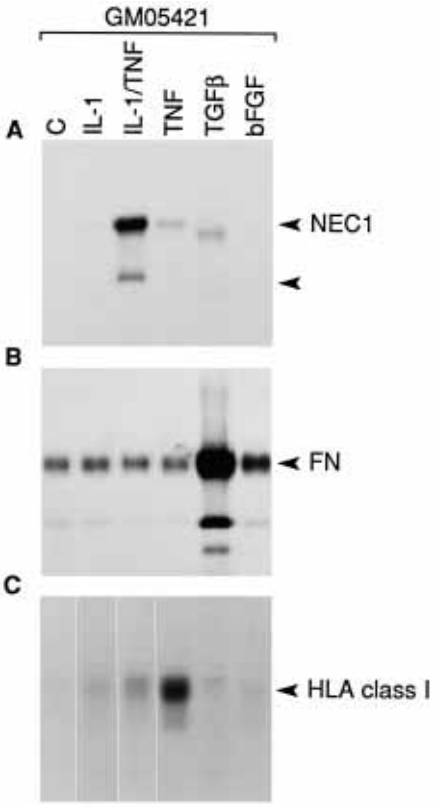

Fig. 7. Differential induction of TN, FN, and HLA class I proteins in GM05421A fetal conjunctival fibroblasts. Confluent cultures (passage 15) grown in MEM-0.5%FBS were Tran^{35}S-labeled in MEM-0.5%FBS alone (C) or MEM-0.5%FBS supplemented with the indicated factors: IL-1α (2 ng/ml), TNFα (50 ng/ml), IL-1α/TNFα, TGFβ (2 ng/ml), or bFGF (25 ng/ml). After 18 hours, supernatants were collected for tests with αNEC1a/b (A) or αFN (B), and NP40 cell extracts were prepared for tests with mAb W6/32 (C). Negative controls with unrelated mouse IgG1 were included in all assays (not shown). The positions of the M_r 250,000 and 180,000 species of TN, the major FN species (M_r 230,000), and HLA class I molecules (M_r 43,000) are indicated on the right. The weak M_r 230,000 band in the TGFβ lane of (A) reflects nonspecific binding of FN.

Several control experiments demonstrated the specificity of TN induction in fetal conjunctival fibroblasts. First, we observed only minor changes in total protein synthesis and cell numbers in treated and untreated cultures, unrelated to the levels of TN production. Second, FN production was generally increased by treatment with TGFβ (Fig. 7B) but was unaffected or even reduced with IL-1, TNFα, and IL-4. Third, the expression of MHC class I antigens, which are present at low levels on unstimulated fetal fibroblasts, was upregulated by TNFα (Fig. 7C) but not by IL-1, IL-4, or TGFβ. Finally, the expression of α1/β1 integrins was selectively increased by IL-1/TNFα, and FAP expression was high in treated and untreated cultures.

Dose response curves for TN induction in GM05421A cells (Fig. 9) showed that IL-1α IL-1α, IL-1β, and IL-4 are active at 0.01 to >10 ng/ml and TNFα is active at 0.1 to >100 ng/ml. In additional tests, we found that IL-1α and TNFα induce TN secretion within 5-8 hours after addition of factors.

Effects of cycloheximide and actinomycin D on GM05421A cells

When GM05421A cells were pretreated with actinomycin D (0.1 μg/ml) or cycloheximide (10 μg/ml) one hour prior to adding IL-1 or TNFα and cultured for 24 hours, total protein synthesis was reduced by only 10-25% but TN induction was completely abolished. This finding indicates that de novo mRNA and protein synthesis is necessary for TN induction.

TN induction in TN^{low} fetal leptomeningeal cells

The FB2, FM3, and FB20 fetal leptomeningeal fibroblast strains produce no or only trace amounts of TN while secreting abundant FN (Table 1; Fig. 10). Solid-phase ELISA tests with FB20 culture supernatants showed that TN levels in serum-supplemented and serum-free 24-hour cultures are <0.02 μg/ml (Table 1) and that none of the following factors induced TN: aFGF, bFGF, TPA, TGFβ, PDGF, EGF, BMP-1, BMP-2, BMP-3, IGF-1, IFNγ, retinol, and retinoic acid. However, a significant increase in TN levels was detected in cultures treated with a combination of IL-1β (or IL-1α), TNFα, and IL-4 (1-2 μg/ml TN at 24 hours), IL-1β and TNFα (0.07 μg/ml TN) or IL-4 alone (0.2-0.7 μg/ml TN). Immunoprecipitation tests identified M_r 250,000 and 180,000 TN proteins in the spent media, detergent cell extracts, and detergent-resistant ECM (Fig. 10) of IL-1/TNFα/IL-4-treated FB20 cultures, but not in untreated or TGFβ-treated control cultures.

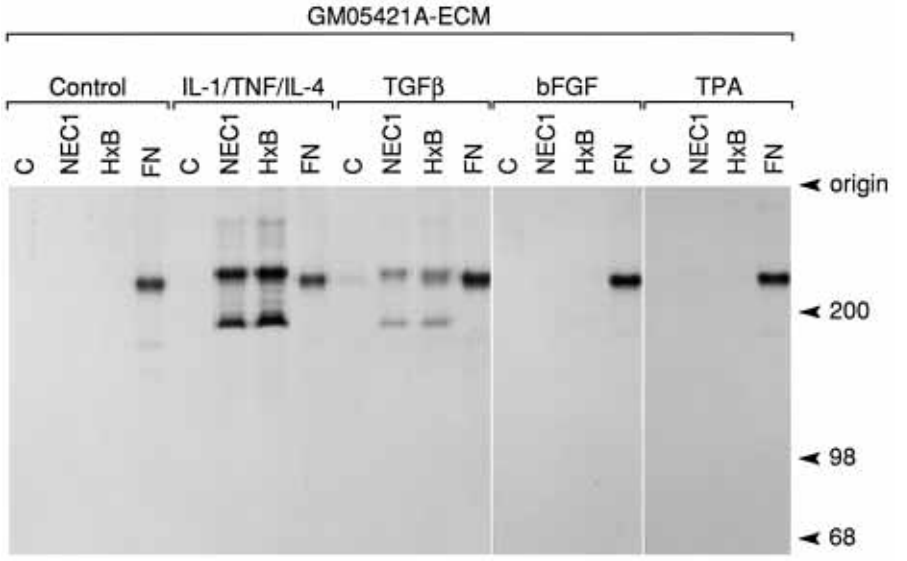

Fig. 8. Immunoprecipitation of TN and FN from the cell-free ECM of GM05421A fetal conjunctival fibroblasts. Fibroblasts (passage 9) grown in MEM-0.5%FBS (control) or MEM-0.5%FBS supplemented with IL-1β (2 ng/ml)/TNFα (50 ng/ml)/IL-4 (1 ng/ml), TGFβ1 (2 ng/ml), or bFGF (10 ng/ml), were Tran^{35}S-labeled. Supernatants were decanted, cell monolayers removed with 0.5% NP-40 buffer, and the cell-free ECM extracted with high-pH buffer (30 mM diethylamine, 1 mM EDTA, pH 11.5) and used for immunoprecipitation tests with negative control mouse IgG1 (C), αNEC1a/b, αHxB, or αFN. The top of the running gel (origin) and the positions of molecular markers ($\times 10^{-3}$) are indicated to the right.

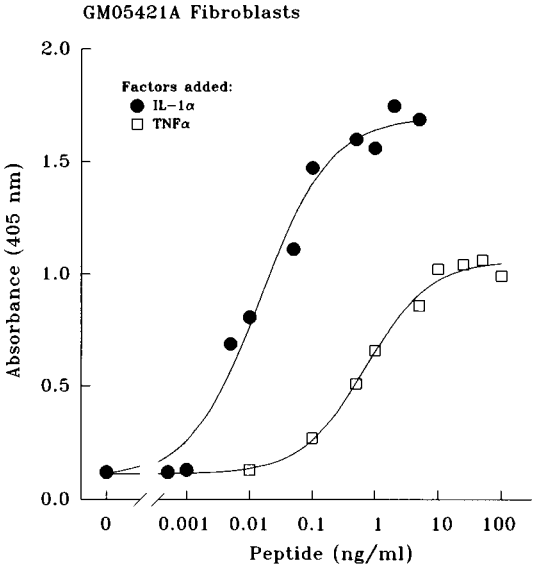

Fig. 9. Dose-response curves for TN induction in GM05421A fetal conjunctival fibroblasts stimulated with IL-1α or TNFα. Confluent cultures were grown for 24 hours in MEM-0.5%FBS or MEM-0.5%FBS supplemented with IL-1α or TNFα, and supernatants were tested with αNEC1a/b by solid-phase ELISA. Background absorbance as determined with negative control mouse IgG1 was 0.08-0.15 in all assays.

Effects of PDGF and TGFβ on fibroblast TN production

Analysis of Hs68 newborn foreskin fibroblasts, which show intermediate levels of basal TN production when grown in MEM-10%FBS (Table 1), indicated distinct roles for PDGF-BB, IL-1/TNFα, and TGFβ in TN regulation. ELISA and immunoprecipitation tests showed that Hs68 cells grown in MEM-10%FBS produce about 0.2-0.7 μg/ml TN in standard 24-hour assays, with an increase to >2 μg/ml upon addition of IL-1β (2 ng/ml)/TNFα (25 ng/ml). Hs68 cultures grown for 24 hours in low-serum media (MEM-0.5%FBS) produced about 15-fold lower levels of TN than MEM-10%FBS cultures, with no significant changes in cell numbers, viability, or FN production. This decrease was reversed by adding either 50 ng/ml PDGF-BB (0.7 μg/ml TN after 24 hours) or a combination of IL-1β (2 ng/ml) and TNFα (25 ng/ml) to the MEM-0.5%FBS media (1.5 μg/ml TN after 24 hours).

TGFβ showed predominantly inhibitory effects on TN production by Hs68 cultures. For example, TGFβ treatment (2 to 10 ng/ml) reduced TN levels up to 20-fold when the cells were grown in either MEM-10%FBS, or MEM-0.5%FBS, or MEM-0.5%FBS supplemented with 50 ng/ml PDGF-BB. However, TGFβ had no or only weakly inhibitory effects on TN production in Hs68 cultures grown in MEM-0.5% with IL-1/TNFα (1.3 to 1.5 μg/ml TN after 24 hours).

DISCUSSION

The results of our study support the idea that the diversity of TN distribution patterns in developing organs (Aufderheide et al., 1987; Aufderheide and Ekblom, 1988; Crossin et al., 1986; Mackie et al., 1987), wound healing (Mackie et al., 1988; Garin-Chesa et al., 1989; Lightner et al., 1989;), nerve repair (Daniloff et al., 1986), skin lesions (Lightner et al., 1989; Garin-Chesa et al., 1989), and cancers (Bourdon et al., 1983; Chiquet-Ehrismann et al., 1986; Garin-Chesa et al., 1989) can be traced to a limited number of simple, cell type-specific patterns of constitutive or inducible TN expression. Contrary to the initial view of TGFβ as the principal regulator of TN in developing and lesional tissues, it seems likely that distinct sets of extrinsic factors modulate TN expression in different cell types. Two lines of evidence support such a modular concept of TN regulation. First, the cell culture studies described in this and previous reports (Pearson et al., 1988; Rettig and Garin-Chesa, 1989; Rettig et al., 1989; Yavin et al., 1991; McCachren and Lightner, 1992; Sharifi et al., 1992; Mackie et al., 1992; Tucker et al., 1993) identify a number of growth factors, cytokines, phorbol ester, and angiotensin II as potential regulators of TN expression, and developing tissues, wounds, inflammatory cells, and cancers are known sources for several

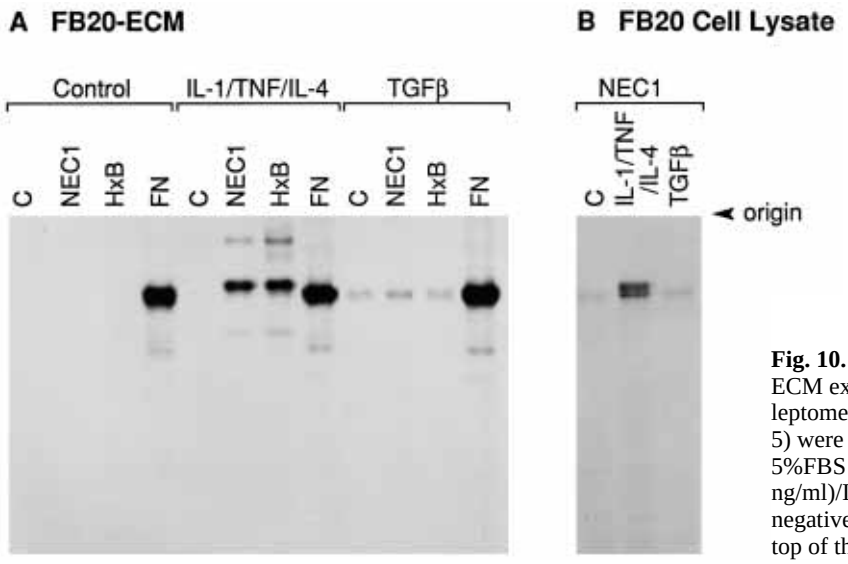

Fig. 10. Immunoprecipitation of TN and FN from high-pH ECM extracts (A) and NP40 cell lysates (B) of FB20 fetal leptomeningeal fibroblasts. Confluent FB20 cultures (passage 5) were Tran^{35}S-labeled in MEM-5%FBS (control) or MEM-5%FBS supplemented with IL-1β (1 ng/ml)/TNFα (50 ng/ml)/IL-4 (1 ng/ml) or TGFβ (2 ng/ml) and tested with negative control mouse IgG1 (C), αNEC1, αHxB, or αFN. The top of the running gel (origin) is indicated to the right.

of these mediators (Basilico and Moscatelli, 1992; Le and Vilcek, 1987; Massague, 1990; Nathan and Sporn, 1991). Second, Jones et al. (1990) have identified an array of putative cis-acting regulatory elements flanking the chicken *TN* gene, including sequences that confer responsiveness to soluble growth and differentiation factors, and these genetic elements may allow for cell type-specific modes of TN regulation.

If TN is inducible by multiple growth and differentiation factors, including some that are broadly expressed in normal tissues, how is the restricted tissue distribution of TN achieved? A possible explanation for this paradox comes from the comparison of TN induction in cells of different cell lineages that express overlapping sets of receptors for growth and differentiation factors. These studies suggest that TN inducibility in a given cell type is not simply controlled by the availability of extrinsic signals and expression of their functionally active cognate receptors but also by intrinsic factors, presumably cell type-specific differences in signalling pathways.

This added level of control for TN expression is illustrated by the effects of FGF and TPA on neural, melanocytic, and fibroblastic cells. In the absence of fetal human neural precursor cells for in vitro analysis, cell lines derived from neuroectodermal tumors, such as PNET, neuroblastoma, and melanoma, and short-term cultures of normal melanocytes have emerged as the only tenable models for studying molecular differentiation and signal transduction pathways in human neuroectoderm-derived cells (Becker et al., 1992; Halaban et al., 1992; Ip et al., 1992; Rettig et al., 1992a; Thomson et al., 1989). Our studies show that FGFs and TPA are potent inducers of TN in PNET cells, with combinations of FGF and TPA producing up to >100-fold increases in TN production. Different FGFs signal through a common set of surface receptors, which explains the similarity of action for aFGF, bFGF, and K-FGF (Basilico and Moscatelli, 1992; Johnson and Williams, 1993). Phorbol esters like TPA exert most of their effects through activation of protein kinase C (PKC) (Nishizuka, 1992), and the PKC-dependent signalling pathways are not generally linked to FGF-induced effects. Both FGF and TPA induce TN expression in PNETs independent of changes in cell proliferation, total protein synthesis, or production of other ECM proteins, while requiring de novo RNA and protein synthesis.

Cultured human melanocytes express functional FGF receptors and require FGF and TPA for survival and proliferation in vitro (Albino et al., 1992; Becker et al., 1992) but they fail to produce TN. Thus, FGF and TPA responsiveness in melanocytes and PNETs differs clearly with regard to TN, most likely due to differences in the FGF- and TPA-activated signalling pathways. The alternative explanation that the signalling pathways are similar but that the TN gene is irreversibly inactivated in melanocytic cells appears less likely since TN expression is detected in fully transformed melanocytes and some melanoma cell lines. Consistent with this idea, we found that FGFs and TPA modulate proliferation and surface antigen expression in fibroblasts but not their TN expression. It remains to be determined at which level of signal transduction PNETs, melanocytes, and fibroblasts differ. Conceivably, TPA- and FGF-dependent second messengers may be linked to disparate 'down-stream' signalling molecules and target genes in different cell types, or more than one signal may be required to activate TN expression, with different combinations of signals being active in different cell types.

Immunohistochemical studies have identified reactive fibroblasts as a major source of TN in wound healing and tumor stroma. As a first step toward establishing the role of extrinsic mediators in fibroblast TN expression, we decided to quantify basal TN production in several normal fibroblast strains, and to monitor TN levels at successive passage levels. Unexpectedly, these preliminary studies revealed up to >100-fold differences in basal TN production for fibroblasts derived from different organ sites and stages of development. These differences were seen even for fibroblasts established synchronously from different organ sites of the same donors; they were stably maintained in culture, and were independent of proliferative activity, total protein synthesis, FN production, and surface antigen expression. Immunohistochemical findings suggest that TN^{high}, TN^{intermediate}, and TN^{low} fetal fibroblast strains may come from tissues with comparable levels of TN expression, rather than being derived from organs with dra-

matically different TN levels in vivo. It is tempting to speculate that fibroblasts in some normal tissues are capable of high-level TN expression, but they do not initiate TN production until the appropriate extrinsic signals become available during development or are provided in vitro with tissue culture-related factors.

The identification of TN^{low} and $TN^{intermediate}$ fibroblast strains has facilitated the search for TN-inducing factors without the need for prior downregulation of basal TN levels through serum-starvation. With this approach we identify TNFα, IL-1α, IL-1β, and IL-4 as the most potent TN inducers in fibroblastic cells. Additional effects were seen for PDGF-BB using serum-starved $TN^{intermediate}$ fibroblasts. Previous studies have shown that TNFα stimulates the growth of fetal fibroblasts and promotes ECM degradation in bone and cartilage (Le and Vilcek, 1989). Since TNFα, IL-1, and IL-4 are present in lesional tissues such as rheumatoid arthritis, granulation tissue of healing wounds, and cancers, all of which show increased TN expression, it appears likely that these cytokines serve additional functions in regulating ECM protein secretion. The synergism between TNFα and IL-1, two factors that bind to structurally distinct cell surface receptors, is consistent with observations in many other test systems in which IL-1 enhances TNFα effects (Le and Vilcek, 1989; Waage and Espevik, 1988). The molecular basis for this cooperativity is not clearly understood, but TNFα and IL-1 may activate a common sphyngomyelin signal transduction pathway (Dressler et al., 1992). Both factors are commonly expressed by the same cells or by different cell types participating in the same biological reactions (Le and Vilcek, 1987; Nathan and Sporn, 1991), suggesting that their cooperativity in vitro may be relevant in vivo also. Precedents for functional interactions between TNFα and IL-4 have also been described, including cooperative and opposing effects. For example, IL-4 potentiates the antiproliferative effect of TNFα on certain tumor cells (Totpal and Aggarwal, 1991). In endothelial cells, IL-4 enhances TNFα-induced VCAM-1 expression but downregulates TNFα-induced ICAM-1 and ELAM-1 expression (Thornhill and Haskard, 1990; Thornhill et al., 1991). Finally, in several hematopoietic cell types, IL-4 inhibits the expression of IL-1, TNFα, and other cytokine genes (Essner et al., 1989).

The effects of TGFβ on TN expression may be more complex than previously thought. In human fibroblasts, TGFβ can augment or reduce expression of TN, depending on the fibroblast strains used and the presence of other factors in the culture system. This ability of TGFβ to function as a molecular 'switch' with opposite effects in different cell types or under different culture conditions has been observed previously (Massagué, 1990; Nathan and Sporn, 1991). It may be due to the fact that TGFβ does not only exert direct effects on gene expression but also modulates other signalling pathways (Battegay et al., 1990). For example, the TN-inducing activity of TGFβ for CEFs was detectable only in serum-starved cultures (Pearson et al., 1988). Since TGFβ appears to exert its effects through at least two functionally distinct cell surface receptors (Chen et al., 1993), it remains to be seen whether positive and negative effects of TGFβ on fibroblast TN expression are mediated by the same receptor species.

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