Molecular cloning and tissue distribution of pig transforming growth factor α

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Transforming growth factor α (TGF α) was originally identified as a product of tumour tissues and transformed cells in culture. Although it is now clear that expression of this factor is not restricted to neoplastic cells, there remains relatively little information about the sites of expression of TGF α in normal tissues. Therefore, an amplified DNA fragment encoding the pig TGF α precursor was cloned by reverse transcription-PCR (RT-PCR) using RNA isolated from normal skin tissue as the template. Nucleotide sequence analysis predicts a 160-residue transmembrane polypeptide that differs from the rat, mouse and

human TGF α precursors at 14, 15 and six sites respectively. The distribution of TGF α mRNA in a wide variety of pig tissues was analysed by RT-PCR, using oligonucleotide primers based on the pig TGF α cDNA sequence. TGF α transcripts were detected in RNA isolated from 17 of the 22 tissues analysed, including four previously unreported sites. Using an antibody raised against a synthetic TGF α peptide, we have immunolocalized TGF α protein to cells within the red pulp of the spleen and to the distal convoluted tubules of the kidney.

INTRODUCTION

Transforming growth factor α (TGF α) is a 50-amino-acid polypeptide that is mitogenic for a variety of cell types (see Massagué, 1990). It is both structurally and functionally related to epidermal growth factor (EGF) and interacts with the same cell-surface receptor (see Fisher and Lakshmanan, 1990). Analyses of the human (Derynck et al., 1984) and rat (Lee et al., 1985; Blasband et al., 1990) TGF α cDNAs indicated that the mature (50 amino acid) polypeptides are derived from larger precursor proteins of 160 and 159 residues respectively. The precursors contain an extracellular domain of around 100 amino acids that includes the mature peptide and an N-terminal signal sequence, a 23-residue hydrophobic transmembrane domain, and a 39-residue cytoplasmic domain. A variety of TGF α species, including the mature form, are released from the transmembrane TGF α precursor by differential proteolytic cleavages in its extracellular domain (Bringman et al., 1987). In the absence of processing, the precursor is also biologically active; co-culture of cells over-expressing the EGF receptor with those expressing high levels of membrane-bound TGF α precursor results in receptor auto-phosphorylation and signal transduction (Wong et al., 1989; Brachmann et al., 1989).

TGF α was originally identified in medium conditioned by mouse-sarcoma-virus-transformed fibroblasts (De Larco and Todaro, 1978), and subsequently in a number of tumours and tumour-derived cell lines (Derynck et al., 1987; Schlegel et al., 1990). Expression of TGF α , however, is not restricted to neoplastic cell types, as TGF α mRNA has been detected during early mammalian development (Werb, 1990; Vaughan et al., 1992a). Additionally, TGF α mRNA and/or protein have been identified in a number of normal adult tissues, including rodent brain (Wilcox and Derynck, 1988; Lazar and Blum, 1992), human keratinocytes (Coffey et al., 1987) and activated human macrophages (Madtes et al., 1988). Despite the potential importance of TGF α in tissue proliferation and differentiation, few systematic studies of the expression of TGF α have been reported.

In the absence of such information, it is difficult to address the question of its physiological function(s). In the current study, we report the nucleotide sequence encoding the pig TGF α precursor and the distribution of its mRNA in a wide range of pig tissues. Furthermore, we have used an anti-TGF α antiserum to localize TGF α protein in a number of tissues.

EXPERIMENTAL

Isolation of RNA

Large White pigs were killed either at a local slaughterhouse by stunning and exsanguination, or at the Institute by intravenous injection of an overdose of barbiturate anaesthetic. Tissue samples were removed and processed immediately or snap-frozen in liquid nitrogen and stored at -70°C until required. Total RNA was isolated by a single-step guanidine thiocyanate method (Chomczynski and Sacchi, 1987).

Oligonucleotide primers

The oligonucleotide primers were synthesized on a Biosearch 8750 four-column DNA synthesizer (Biosearch, San Rafael, CA, U.S.A.). TGF α primers were designed according to conserved regions of the rat (Blasband et al., 1990) and human (Derynck et al., 1984) cDNAs. For cloning purposes, each primer contained an 8 bp 5'-extension (5'-CGGGATCC-3') incorporating a *Bam*HI restriction enzyme site. The upstream primer [5'-GC(T/C)CG(T/G)AA(A/G)ATGGTCCC-3'] represented nucleotides 26 to 43 of the human sequence (Derynck et al., 1984), while the downstream primer [5'-CCACCTGGCCAAA-(T/C)TCCT-3'] was the reverse complement of nucleotides 529 to 547. This primer pair predicts a 538 bp fragment. A second pair of primers was used for the tissue distribution analysis of TGF α mRNA. The upstream primer (5'-TGCCAGATT-CCCACAGT-3') was identical with nucleotides 148 to 165 of the cloned pig cDNA, while the downstream primer (5'-TGTATC-

AGCACACAGGTG-3') was the reverse complement of nucleotides 357 to 374. This primer pair predicts a 243 bp fragment.

The β -actin upstream primer (5'-CTACAATGAGCTGCG-TGTGG-3') is identical to nucleotides 192 to 211 of the mouse β -actin cDNA (Tokunaga et al., 1986), while the downstream primer (5'-TAGCTCTTCTCCAGGGAGGA-3') was the reverse complement of nucleotides 622 to 641. This primer pair predicts a 450 bp fragment and spans a known intron, so that amplification of actin mRNA (as cDNA) may be distinguished from amplification of any contaminating genomic DNA.

Reverse transcription-PCR (RT-PCR)

First-strand cDNA was synthesized from total RNA (1 μ g) using a 'First-Strand cDNA Synthesis' kit (Pharmacia) and 50 pmol of the appropriate downstream primer. Reaction products were precipitated with 0.1 vol. of 4 M NaCl and 2.5 vol. of ethanol, and resuspended in 10 μ l of water. One-tenth of the cDNA was subjected to PCR amplification using a Techne PHC-1 programmable Dri-Block (Techne Ltd., Duxford, Cambs., U.K.). The amplification profile comprised 35 cycles: at 94 °C for 0.3 min (dissociation), 55 °C for 0.5 min (annealing) and 72 °C for 1.0 min (extension). The final cycle included a further 5 min at 72 °C for complete strand extension. Each reaction mix contained 5 μ l of cDNA, 50 pmol of each primer, 5 μ l of 10 $\times$ PCR buffer (Promega), 2 μ l of 5 mM dNTPs and water to a final volume of 50 μ l. After overlaying with 50 μ l of light mineral oil (Sigma), the reactions were heated to 94 °C for 5 min before the addition of 2.5 units of *Taq* polymerase (Promega). All RT-PCR reactions were performed at least twice for each individual RNA sample.

Southern-blot analysis

Digested pig genomic DNA (10 μ g) was separated by electrophoresis on a 0.9% agarose gel and transferred to a nylon membrane (Hybond-N; Amersham, Bucks., U.K.). Probe DNA was radiolabelled by the random-primed DNA synthesis method (Feinberg and Vogelstein, 1983). High-stringency hybridization and washing conditions were employed as previously described (Pascall and Brown, 1988). Hybridizing sequences were detected by autoradiography using Fuji RX X-ray film.

DNA sequencing

PCR products were purified from low-melting-point agarose gels and subcloned into pBluescribe-M13⁺ (Vector Cloning Systems). Recombinant double-stranded plasmid DNA was prepared using a CsCl-based 'miniprep' procedure (Saunders and Burke, 1990) and sequenced using a 'Sequenase Version 2.0' DNA sequencing kit (United States Biochemical).

Immunohistochemical staining of tissue TGF α

The affinity-purified rabbit polyclonal antiserum 26T, raised against the 17 C-terminal amino acids of rat TGF α , was a gift from Dr. W. J. Gullick. Over these 17 amino acids, there is only a single difference between the rat and pig sequences (see Figure 3 below). Tissues were snap-frozen in isopentane cooled with solid CO₂. Serial cryostat sections (8 μ m) were collected on aminopropyltriethoxysilane-coated glass slides and allowed to air-dry for 2 h. Immunocytochemical staining of sections was performed as previously described (Vaughan et al., 1991), except that the anti-TGF α antiserum was used at a dilution of 30 μ g/ml and the sections were incubated with the primary antiserum for

1 h. In competition studies, 100 μ l of anti-TGF α antiserum (at the working dilution) was preabsorbed with 10 μ g of free TGF α peptide (26T; from Dr. W. J. Gullick) or 50 μ g of recombinant pig EGF for 30 min at room temperature before performing the immunohistochemistry.

RESULTS AND DISCUSSION

Although expression of TGF α mRNA was initially considered to be associated with neoplastic transformation (Derynck et al., 1987), subsequent studies have demonstrated that the factor is also expressed by some non-transformed cells and tissues such as rodent brain (Wilcox and Derynck, 1988; Lazar and Blum, 1992) and human skin (Coffey et al., 1987). However, systematic studies of TGF α mRNA expression have not been reported, presumably because of the low levels of expression generally observed (for example, see Rappolee et al., 1988b). We have therefore used the highly sensitive technique of RT-PCR, in conjunction with oligonucleotide primers specific for TGF α , to investigate the expression of TGF α mRNA by a wide variety of pig tissues.

Cloning and nucleotide sequence of pig TGF α cDNA

A mouse TGF α cDNA (encoding the mature form of TGF α ; Vaughan et al., 1992b) was used to probe restriction endonuclease digests of pig genomic DNA. Under high-stringency hybridization and washing conditions, one hybridizing band was detectable in each digest (Figure 1), indicating that TGF α is present as a single-copy gene in the pig genome, as previously reported for the rat (Lee et al., 1985).

In order to allow the design of homologous PCR primers, it was necessary to determine the pig TGF α cDNA sequence. We

Figure 1 Southern blot of pig genomic DNA

Pig genomic DNA (10 μ g/lane) was digested with *Hind*III (lane 1), *Bam*HI (lane 2), *Xba*I (lane 3) or *Bgl*II (lane 4), electrophoresed through a 0.9% agarose gel and transferred to a nylon membrane. The blot was probed with a 243-bp radiolabelled mouse TGF α cDNA (encoding the majority of the mature peptide), washed under conditions of high stringency and exposed to X-ray film for 5 days with an intensifying screen at -70 °C. The positions of the *Hind*III-digested λ DNA markers (in kb) are indicated.

therefore isolated total RNA from normal adult pig skin and used this as a template for RT-PCR in conjunction with oligonucleotide primers based on the human TGF α cDNA sequence. On amplification, a single faint band of the predicted size (538 bp) was observed on an ethidium bromide-stained 1% (w/v) agarose gel (data not shown). No amplification products were detected when either the cDNA or one of the primers was omitted from the PCR, or when the cDNA was replaced with 50 ng of pig genomic DNA. The amplified products from three such RT-PCRs were cloned and sequenced to eliminate any errors that may have been incorporated by the *Taq* polymerase.

The nucleotide sequence of the pig TGF α precursor (Figure 2) predicts a 160-residue membrane-spanning polypeptide. The deduced amino acid sequence differs from that of the rat, mouse and human TGF α precursors at only 14, 15 and six sites respectively (Figure 3). This conservation of TGF α precursor sequence between species contrasts markedly with the considerable species variation observed in the sequences of the related factor, EGF (Burgess, 1989; Pascall et al., 1991). Interestingly, a high degree of TGF α sequence conservation between species is found not only in the extracellular domain but also in the predicted transmembrane and cytoplasmic domains which, when the four sequences are compared, shows only two conservative changes over 62 amino acids (Figure 3). This high degree of sequence conservation between species suggests that the cytoplasmic domain of the precursor has functional significance. Indeed, a function for the C-terminal valine has recently been proposed. Using a series of deletion constructs spanning the cytoplasmic domain, Bosenberg et al. (1992) showed that this amino acid was required for cleavage in the extracellular domain of the precursor in response to various activators, although the mechanisms involved remain to be established.

Tissue distribution of TGF α mRNA

Total RNA isolated from 22 tissues was analysed for TGF α expression using RT-PCR and oligonucleotide primers based on the pig TGF α cDNA sequence. An amplified product of the predicted size (243 bp) was clearly detectable on an ethidium bromide-stained agarose gel when RNA isolated from kidney, duodenum, pancreas, spleen, lung, thyroid, skin, prostate, seminal vesicles, midbrain or cerebellum was used as the template for the RT-PCR (Figure 4). When RNA isolated from the bulbourethral gland, testis, female submaxillary gland, liver, cerebral cortex or hypothalamus was used as the template, only a very weakly staining amplified product was observed (Figure 4), suggesting lower levels of TGF α mRNA present in these tissues. The identity of the 243 bp product amplified from two of the tissues (lung and skin) was confirmed as being derived from TGF α mRNA by nucleotide sequencing. No TGF α -specific amplified products were detected when total RNA isolated from heart, lymph node, male submaxillary gland, pituitary or olfactory bulb was used as the template for the RT-PCR (Figure 4). As a control for RNA integrity, the same RNA samples were analysed by RT-PCR for β -actin expression. Using primers specific for β -actin, all samples gave an amplified product of the predicted size (450 bp; Figure 4).

In man, TGF α is found throughout the epidermis of normal skin (Coffey et al., 1987). We have now extended this observation to a second species and identified TGF α transcripts in normal pig skin. TGF α in human skin has been found to be overexpressed in psoriasis (Elder et al., 1989), to promote wound healing and repair (Schultz et al., 1991), and to stimulate keratinocyte-cell growth and migration (Barrandon and Green, 1987). More

Figure 2 Nucleotide sequence of pig TGF α precursor

A pig TGF α cDNA was isolated by RT-PCR using TGF α -specific primers and total RNA isolated from normal adult pig skin as the template as described in the Experimental section. Amplified products from three such RT-PCRs were cloned and sequenced to eliminate any errors that may have been incorporated by the *Taq* polymerase. The deduced amino acid sequence is shown above the nucleotide sequence. The stop codon is indicated by an asterisk. The TGF α -specific oligonucleotide primers used to amplify first-strand cDNA from normal adult pig skin are underlined.

Figure 3 Comparison of TGF α precursor sequences

The pig TGF α precursor amino acid sequence is compared with those of rat (Blasband et al., 1990), mouse (Vaughan et al., 1992b) and human (Derynck et al., 1984) sequences; only differences are shown. The gap at position 32 in the rat and mouse sequences indicates the absence of a corresponding amino acid. The underlined 50- and 23-residue sequences represent the mature peptide and putative transmembrane domain respectively. The upstream primer used to clone the pig TGF α sequence included nucleotide sequence covering the first three N-terminal amino acids of the precursor. While these three amino acids are likely to be conserved in the pig, we cannot be absolutely certain of their identity.

recently, studies using transgenic mice have suggested that the physiological function of TGF α in skin is in the control of epidermal thickness during development and differentiation

Figure 4 Distribution of TGF α mRNA in pig tissues

Total RNA was isolated from seminal vesicle (lane 1), prostate (2), bulbourethral gland (3), testis (4), skin (5), submaxillary gland (female, 6), submaxillary gland (male, 7), lymph node (8), thyroid (9), heart (10), lung (11), spleen (12), liver (13), pancreas (14), neonatal pancreas (15), duodenum (16), neonatal duodenum (17), kidney (18), neonatal kidney (19), pituitary (20), olfactory bulb (21), hypothalamus (22), cerebral cortex (23), cerebellum (24) and midbrain (25), submitted to RT-PCR using primers specific for (a) TGF α and (b) β -actin, electrophoresed through 1.5% (w/v) agarose gels and stained with ethidium bromide. The predicted sizes of the amplified products for TGF α (243 bp) and β -actin (450 bp) are indicated. Molecular-mass markers (m) are a 100 bp DNA ladder (Gibco BRL).

(Vassar and Fuchs, 1991). Future studies, perhaps using pigs as an experimental model, may help to define more clearly the autocrine/paracrine functions of TGF α in this tissue.

Of the central nervous system tissues analysed, the midbrain, cerebellum, cerebral cortex and hypothalamus were found to express TGF α mRNA, although the final levels of amplified product were lower for cerebral cortex and hypothalamus (Figure 4). Despite the use of a highly sensitive procedure, we were unable to detect TGF α expression in pig olfactory bulb or pituitary. The latter result is somewhat surprising as a TGF α polypeptide has been identified and purified from bovine anterior-pituitary-derived cells in culture (Samsoondar et al., 1986; Kobrin et al., 1986). Whether this discrepancy reflects species variation, the sequestering of TGF α protein by the bovine pituitary, or a difference in the behaviour of pituitary cells in culture from those *in vivo*, remains to be established. Although TGF α is not generally considered to be a neurotrophic or chemotactic factor, its expression in the central nervous system, together with functional EGF receptors (Faundez et al., 1992), suggests that this growth factor may have a function in mammalian neurobiology. In this regard, the related factor, EGF, has recently been shown to induce the differentiation of neuroepithelial stem cells of the mouse striatum into neurons and astrocytes *in vitro* (Reynolds and Weiss, 1992).

Expression of TGF α or TGF α mRNA by kidney (Brown et al., 1990), duodenum (Kommos et al., 1990) and pancreas (Barton et al., 1991) has previously been reported for human or rat tissues. We confirm here that these tissues also express TGF α mRNA in adult pigs. In addition, we have extended these findings by showing TGF α expression in the same tissues of neonatal animals (Figure 4). While there was no apparent difference in TGF α expression between adult and neonate pancreas or kidney, the results reveal an apparently higher level of TGF α mRNA in the neonatal duodenum compared with that

of the mature animal. However, further analysis using more quantitative assays will be required to confirm this observation.

To our knowledge, this study represents the first demonstration of TGF α expression in male accessory organs, namely the seminal vesicles and prostate. Since EGF receptors have been immunolocalized to the basal cells of the prostate (Fukuyama and Shimizu, 1991), these results suggest that TGF α may have an autocrine/paracrine function in normal prostate and could be involved in the development of prostatic hyperplasia. We were also able to detect TGF α mRNA at two other previously unreported sites: the thyroid and spleen. The spleen, however, contains macrophages, and these cells have been demonstrated to express TGF α transcripts (Rappolee et al., 1988a). It is therefore possible that the signal detected in the spleen by RT-PCR may be macrophage-derived. Although TGF α immunoreactivity has been previously observed in human thyroid tumours and in thyroiditis, no immunostaining was seen in normal thyroid (Lemoine et al., 1991). However, the related factor, EGF, has been identified in human (Kajikawa et al., 1991) and mouse (Dagogo-Jack et al., 1986) thyroid tissue. EGF and TGF α have similar functions and it is therefore possible that TGF α could substitute for EGF in the pig thyroid. In addition, specific high-affinity EGF receptors have been demonstrated on pig thyroid membranes (Atkinson et al., 1983), suggesting that TGF α could act locally. However, since the mature thyroid is largely mitotically inactive, endogenously synthesized TGF α is more likely to be involved in the control of thyroid function rather than in growth and development.

Immunohistochemical localization of TGF α in the kidney and spleen

In order to begin to identify cellular sites of TGF α synthesis within expressing tissues, cryostat sections cut from selected

Figure 5 Immunohistochemical localization of TGF α in spleen and kidney

Staining was performed as described in the Experimental section. Immunoreactivity was detected in (a) spleen and (c) kidney. No specific staining was observed when the TGF α antiserum was preabsorbed with TGF α peptide before use (b, spleen; d, kidney). Scale bars represent 50 μ m.

adult tissues (kidney, seminal vesicles, prostate, thyroid and spleen) were immunostained using an antiserum raised against the 17 C-terminal amino acids of rat TGF α (Barton et al., 1991). We were unable to identify the sites of TGF α synthesis in the seminal vesicles, prostate or thyroid. This could be due to a low level of expression throughout the abundant epithelial cells of these tissues. In the spleen, we observed intense cytoplasmic immunostaining of a small number of cells within the red pulp (Figure 5a). Although we are currently unable to conclusively identify the stained cell type, one possibility is that they are macrophages which are present in red pulp and are known to express TGF α mRNA (Rappolee et al., 1988a). The immunoreactivity was reproducible and specific, as no staining was seen on serial sections when the primary antibody was either omitted or was preabsorbed with TGF α peptide (Figure 5b).

In the kidney, we observed intense staining of the distal convoluted tubules, with no immunoreactivity in either the proximal tubules or the glomeruli (Figure 5c). Interestingly, the distal tubules are also a major site of synthesis of the related factor, EGF, in all species studied, including the pig (Vaughan et al., 1991). However, it seems very unlikely that the observed TGF α immunostaining in these tubules is a result of cross-reaction with EGF, for a number of reasons. First, the TGF α antiserum used in this study was raised against the 17 C-terminal

amino acids of rat TGF α . While there is only a single difference between the rat and pig TGF α sequences over these 17 amino acids, there are seven changes in the comparable region of the pig EGF sequence (Pascall et al., 1991). Secondly, in contrast with that observed for TGF α (Figure 5), EGF immunostaining appears cytoplasmic with no membrane accentuation (Vaughan et al., 1991). Finally, while preabsorption of the anti-TGF α antiserum with recombinant pig EGF did not affect either the pattern or intensity of staining (results not shown), no staining was observed when the antiserum was preabsorbed with TGF α peptide (Figure 5d). To our knowledge, this is the first demonstration that TGF α is, like EGF, synthesized by the distal convoluted tubules of the kidney.

Recent studies using polarized Madin–Darby canine kidney cells have demonstrated that EGF receptors localize to the basolateral membrane of kidney epithelial cells (Brandli et al., 1991). Binding of locally synthesized EGF or TGF α to these receptors may affect renal function. Indeed, exogenously administered EGF or TGF α promotes kidney tubule formation in matrigel cultures (Taub et al., 1990), and EGF can stimulate repair of damaged areas of epithelium along the nephron after ischaemic injury (Humes et al., 1989). In addition, EGF can inhibit sodium reabsorption from isolated microperfused collecting tubules (Vehaskari et al., 1989), and both TGF α and EGF have been shown to decrease sodium-dependent phosphate transport in cultured renal epithelial cells (Pizurki et al., 1990). As both EGF and TGF α are synthesized by the distal convoluted tubules of the kidney, these results therefore raise the possibility that these factors function as local paracrine/autocrine regulators of renal tubule transport and/or regeneration.

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