Expression of transforming growth factor β (TGFβ) receptors and expression of TGFβα, TGFβ, and TGFβ in human small cell lung cancer cell lines

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Summary A panel of 21 small cell lung cancer cell (SCLC) lines were examined for the presence of Transforming growth factor β receptors (TGFβ-r) and the expression of TGFβ mRNAs. By the radioreceptor assay we found high affinity receptors to be expressed in six cell lines. Scatchard analysis of the binding data demonstrated that the cells bound between 4.5 and 27.5 fmol mg⁻¹ protein with a Kd ranging from 16 to 40 pM. TGFβ binding to the receptors was confirmed by cross-linking TGFβ to the TGFβ-r. Three classes of TGFβ-r were demonstrated, type I and type II receptors with M(r) = 65,000 and 90,000 and the betaglycan (type III) with M(r) = 280,000. Northern blotting showed expression of TGFβ mRNA in ten, TGFβ mRNA in two and TGFβ mRNA in seven cell lines. Our results provide, for the first time, evidence that a large proportion of a broad panel of SCLC cell lines express TGFβ receptors and also produce TGFβ mRNAs.

The TGFβ family consists of several members of structurally related proteins. The first member of this family to be cloned was TGFβ (Derynck et al., 1985). To date three other members have been cloned and described TGFβ (Miller et al., 1989b; Madisen et al., 1988; Martin et al., 1987) from murine and human source. TGFβ (Miller et al., 1989a) from murine source and TGFβ from chicken embryonic tissue (Jakowlew et al., 1988). These members form a complex network of interacting ligands. The role for each of these has not been clearly elucidated but the expression pattern in the mouse embryo suggest a role in differentiated role in embryogenesis (Pelton et al., 1991). The TGFβ family of peptides exerts both stimulatory and inhibitory effects depending on cell type examined (Barnard et al., 1990).

Receptors for TGFβ have been demonstrated in a variety of normal cells of both epithelial and mesenchymal origin as well as in several malignancies (Frolik et al., 1984; Tucker et al., 1984; Massagué & Like, 1985; Wakefield, 1987). At present five distinct TGFβ-r have been identified, type I (Mr = 60-70,000), type II (85-110,000), type III (200-400,000), type IV (60,000) and type V (40,000). The type II and III receptors have recently been cloned (Lin et al., 1992, Wang et al., 1992). In addition a TGFβ binding protein (150,000 and 180,000) has been described, which binds TGFβ, but not TGFβ (MacKay & Danielpour, 1991). The type I and II receptors are the most probable candidates as the mediator of the signal induced by TGFβ (Boyd & Massagué, 1989; Laiho et al., 1990). The type III receptor is believed to be a surface associated proteoglycan, which binds TGFβ and ultimately releases it (Andres et al., 1989) or is internalised with TGFβ (Massagué, 1990). The type IV receptor has been identified in pituitary cells, but its function has not been established (Cheifetz et al., 1988). The function of the type V receptor, which has been purified from bovine liver, is unclear at present (O'Grady et al., 1991). Several malignancies have been screened for the presence of TGFβ-r, but in human lung cancer the data is very sparse. A few studies have demonstrated that TGFβ mRNA was expressed in only non-SCLC (NSCLC) cell lines (Söderdahl et al., 1988; Derynck et al., 1987; Bergh, 1988). In another study all of ten SCLC cell lines examined were found to be TGFβ mRNA negative (Lagadec et al., 1991). In these studies the TGFβ isoform investigated was not specified, but most probably it was TGFβ, mRNA.

These data are the basis for the concept that only NSCLC cell lines can produce TGFβ (for review, see Pelton & Moses, 1990).

In the present study we have examined the presence of TGFβ-r and the production of TGFβ mRNA in a panel of 21 SCLC cell lines established in five different laboratories. The results showed that a relatively high proportion of SCLC cell lines carried high affinity TGFβ-r and expressed TGFβ mRNA. Coexpression of TGFβ-r and TGFβ was found in six SCLC cell lines.

Materials and methods

Cell lines

SCLC cell lines were cultured in 150 cm² flasks at 37°C under standard conditions in medium containing 10% foetal calf serum (Flow Laboratories, Irvine, Scotland) without antibiotics. We have previously reported in detail the growth morphology and tissue culture media for these cell lines (Damstrup et al., 1992). Twenty-one SCLC cell lines established from 17 patients in five different laboratories were examined. Eight cell lines were established at Dartmouth Medical School, Hanover, NH, USA (DMS), seven cell lines were established at Gronningen Lung Cancer Center, Groningen, the Netherlands (GLC), two cell lines were established at the National Cancer Institute, Bethesda, MD, USA (NCI), two cell lines were established in Marburg, Germany (24H and 86M1), and two cell lines were established in our own laboratory Copenhagen, Denmark (CPH). The origin and establishment of the cell lines has been described elsewhere (Pettengill et al., 1980; Carney et al., 1985; de Leij et al., 1985; Bepler et al., 1987; Berendsen et al., 1988; Engelholm et al., 1986). AKR-2B, a mouse fibroblast cell line, which previously has been reported TGFβ-r positive (Tucker et al., 1984) was cultured in Eagle's minimal essential medium (Flow laboratory) supplemented with 10% foetal calf serum, and used as a positive control for TGFβ binding. This cell line was kindly provided by Professor H.L. Moses, Vanderbilt, University, Tennessee. All cell lines were routinely checked for, and found free of, mycoplasma infection.

Cells growing as monolayer cultures were assayed in 35 mm 6-well tissue dishes for radioreceptor assays. Cells were subcultured and used within 24 h of plating. Cells growing as floating aggregates were subcultured and assayed in microfuge tubes within 24 h of subculturing.
Growth factors

Porcine TGFβ, was purchased from British Biotechnology Ltd, Oxford, England and/or was a gift from Bristol-Meyers-Squibb, Pharmaceutical Research Institute, Seattle, USA. Human recombinant EGF and TGFα was purchased from Bissendorf Biochemicals, Hannover, Germany.

TGFβ with a specific activity of 100–180 μCi μg−1 (2.5–4.5 Ci mmol−1), was purchased from New England Nuclear, Boston, USA. The binding activity of 125I-labelled TGFβ was checked at regular intervals using the positive control cell line AKR-2B. The 125I-labelled TGFβ was used within 4 weeks of fresh lot date.

Radioceptor assay

The procedure has been described previously (Massagué & Liker, 1985; Massagué, 1987). Cells growing as monolayer cultures were plated in 35 mm-6 well dishes, usually at 2–5 x 10⁴ cells per well, the day before experiments were performed. The cells were washed for 60 min with binding buffer (128 mM NaCl, 5 mM KCl, 5 mM MgSO₄, 1.2 mM CaCl₂, 50 mM HEPES, pH 7.5 and 2% BSA). After washing, the cells were incubated with 5–10 pM 125I-labelled TGFβ and increasing levels of native unlabelled TGFβ ranging from 0.1 to 2000 pM, the volume of incubation being adjusted to 1 ml. After 2 h incubation at 20°C the reaction was stopped by washing the plates three times with ice cold binding buffer without albumin. After the final wash, the cells were solubilised in solubilisation buffer (128 mM NaCl, 0.25 mM EDTA, 0.5 mM Tris, pH 7.5 and 1% v/v Triton X-100). An aliquot of the supernatant was counted in a Beckmann II gamma counter (70% efficiency). Protein concentration was determined with the BCA protein kit (Pierce Europe, B.V., Oud Beijerland, The Netherlands) (Smith et al., 1985). Cells growing as floating aggregates or cells easily detectable were assayed, as single cell suspensions, in 1.5 ml sigmacote (Pierce) treated microcentrifuge tubes. Viability after obtaining a single cell suspension, assessed by trypan blue exclusion test, was 90–95%. After incubation the reaction was stopped by centrifuging at 5,500 g for 3 min and the cell pellet was resuspended three times in ice cold binding buffer without albumin. After the final wash, the cell pellet was solubilised as above. Maximal binding (Bmax) was calculated as femtomol mg−1 protein by Scatchard analysis of the binding data (Scatchard, 1949). Specificity of the binding was determined in specificity experiments with TGFβ, EGF and TGFα as the displacing agents. The displacing agents were added at the same time as the 125I-labelled TGFβ.

Cross-linking

Washed single cell (2–5 x 10⁴) suspensions were incubated with 40 pM 125I-labelled TGFβ in the presence or absence of a 100-fold excess of unlabelled TGFβ. The incubation proceeded for 4 h at 4°C. After the final wash, the cell pellet was resuspended in 950 μl binding buffer without BSA before 50 μl of 5 mM cross-linking agent disuccinimidyl (DSS) (Pierce, France), freshly dissolved in DMSO, was added. The cross-linking reaction proceeded for 15 min at 4°C and was stopped by centrifuging and washing the pellet in a Tris-containing buffer. Finally the cell pellet was resuspending in 80 μl solubilisation buffer, 10 μl cocktail 1 and 10 μl cocktail 2 as described earlier (Massagué, 1987). The resulting supernatant was boiled for 5 min in sample buffer with 50 mM dithiothreitol (Pierce). One hundred μg protein/lane was run on a 5, 7 or 10%, 8 x 16 cm SDS-PAGE gel. After staining with Coomasie brilliant blue and destaining, the dried gel was exposed to an X-ray film (Amersham) with an intensifying screen at ~80°C.

Northern blotting

RNA was extracted by the single-step acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski & Sacchi, 1987). Ten μg total RNA samples were electrophoresed through denaturing agarose gels containing 2.2 M formaldehyde, and transferred to nylon membranes (GeneScreen Plus, NEN DuPont) as recommended by the supplier. Radio-labeled probes were prepared by the random priming method (Feinberg & Vogelstein, 1983) using [α-32P]dCTP and a commercial kit (both from Amersham). The blots were sequentially hybridised with human probes for TGFβ and for β-actin. The probes for TGFβ were a 2.0 kb full length cDNA (Kasid et al., 1988) obtained from the American Type Culture Collection (No. 59954) and a 267 bp fragment spanning nucleotides 1773–2040. The probe for TGFα mRNA was a 442 bp murine fragment of the plasmid pmTGFβ2-9a (Miller et al., 1989b). The TGFβ probe was a 609 bp murine fragment of the plasmid pmTGFβ-Iib (Miller et al., 1989a). The TGFβ and TGFβ probes were obtained from Professor H.L. Moses, Vanderbilt University. RNA extracted from murine heart and lung was used as positive controls. The β-actin probe was a 2.1 kb BamHI fragment of the plasmid pHF8a-I (Gunning et al., 1983). The membranes were pre-hybridised, hybridised and washed as recommended by the supplier, and exposed to an X-ray film at ~80°C with an intensifying screen.

Results

Receptor binding studies

Saturation of the receptors were reached with a TGFβ concentration in the range of 50 to 100 pM (exemplified in Figure 1). Non-specific binding, defined as the cell associated radioactivity in the presence of a large excess of unlabelled TGFβ, was relatively high but was an inverse function of binding capacity. 23% in AKR-2B with a Bmax of 70 femtomol mg−1 protein and 70% in GLC 19, assayed as a single cell suspension, with a Bmax of 4.5 femtomol mg−1 protein. This relationship has also been described in other cell types (Massagué, 1987). As about half the cell lines grew as floating aggregates and half as monolayer cultures, we chose to relate all binding data to protein concentration. Scatchard analysis of the binding data showed that cells bound between 5 and 27 fmol mg−1 protein with a Kd of 16–40 pm (Table 1).

The specificity of the TGFβ-ligand binding was determined using different displacing agents. The specificity of 125I-labelled TGFβ binding to GLC 3 is shown in Figure 2. It was found that EGF and TGFα, which both binds to the EGF-receptor (Carpenter et al., 1983) did not displace 125I-labelled TGFβ. For all cell lines tested and found positive in the radioceptor assay, binding was in all cases specific and saturable.

Cross-linking studies

TGFβ binding to the receptors was further visualised by cross-linking the ligand-receptor complex with DSS. Figure 3 illustrates the affinity labelling results of eight SCCL-cell lines. Following electrophoresis on a SDS-PAGE gel, specific TGFβ binding was seen as bands with calculated M₀ of 65,000, 90,000 and 280,000; these bands correspond to the type I, II and III TGFβ receptors. The presence of excess unlabelled TGFβ resulted in the disappearance of these bands, demonstrating that the binding was specific. Seven SCCL-cell lines were TGFβ positive in the affinity labelling experiments (Table 1).

Northern blotting studies

The cell lines were examined for the production of TGFβ mRNA. Figure 4a illustrates a Northern blot analysis of 20 cell lines using the full length TGFβ cDNA probe. The TGFβ mRNA is seen as a band of approximately 2.5 kb. Results using the 267 bp TGFβ fragment was similar (data not shown). Nineteen cell lines were hybridised with probes
for TGFβ1 and TGFβ2. In two cell lines (Figure 4b) TGFβ1 mRNA was detected as a faint band of 3.9 kb, TGFβ2 mRNA, size approximately 3.5 kb, was found in seven cell lines (Figure 4c). Blots were rehybridised with β-actin probe to demonstrate equal loading in all lanes. The intensity of staining with the TGFβ probe therefore semiquantifies the TGFβ mRNA content.

The results for all binding data, affinity labelling and Northern blot analysis are summarised in Table 1. Six of the TGFβ-r positive cell lines also expressed TGFβ (Table 1).

### Discussion

This is to our knowledge the first study in which a broad panel of SCLC cell lines has been studied for the presence of TGFβ receptors and the expression of TGFβ mRNA. The panel of SCLC cell lines, established in five different laboratories and cultured in different media, is probably a representative cross-section of SCLC cell lines. In order to standardise our radioreceptor assay, we carried out the normal time- and temperature course experiments as described.
Figure 3  Affinity labelling of the TGFβ receptor. Cells were incubated with $^{125}$I-labelled TGFβ as described in Materials and methods, the receptor complex was cross-linked with DSS and size fractionated on either a, 5% SDS-PAGE gel to demonstrate the high molecular weight betaglycan (type III receptor) or b, 10% gel to demonstrate the receptor type I and II. c, 7% gel for 3 GLC cell lines. For each cell line two lanes were run: − 40 pM $^{125}$I-labelled TGFβ; + 100-fold excess of unlabelled TGFβ. Molecular weight standards from Bio-Rad were co-electroforesed. Roman numerals indicates the type I and II TGFβ-r. Arrow indicate the TGFβ type III receptor.

elsewhere (Frolik et al., 1984; Tucker et al., 1984), and found that the binding was stable for a prolonged period of time at 20°C. Furthermore we found that the receptors could not be demonstrated if the protein concentration was lower than 150 μl ml$^{-1}$. We have previously demonstrated, in the same cell lines, studying the EGF-receptor that this critical protein level was also required to detect the EGF-receptor (Damstrup et al., 1992). Studies on other receptor systems such as the estrogen receptor has also demonstrated this critical protein limit (Skovgaard Poulsen, 1981). Therefore, to avoid underestimating binding capacity or falsely classify a SCLC cell line as TGFβ-r negative, we only drew conclusions on the TGFβ-receptor state in a cell line if the protein concentration was in the range of 200–600 μg ml$^{-1}$.

Analysis of the binding data demonstrated that Scatchard plots in some cell lines were curved near saturation of the receptors. However, it was not possible, with the ligand program developed by Munson and Rodbard (Munson & Rodbard, 1980), to resolve the Scatchard plots into two or more compartments. Other investigators have, in normal rat
kidney cell (NRK), also only demonstrated one class of TGFβ receptors, despite the fact that cross-linking studies with NRK cells have demonstrated that these cells express type I, II and III receptors (Wakefield, 1987; Massagué & Like, 1985; Segarini et al., 1987). Resolving the data with a single class receptor from the linear part of the Scatchard plot demonstrated high affinity receptors in six SCLC cell lines (Figure 1, Table I). The dissociation constant was in all cases characteristic for TGFβ binding (Massagué, 1987; Wakefield, 1987). Maximal binding varied from 4.5 to 27.5 fmol mg⁻¹ protein. Binding of 125I-labelled TGFβ1 to the positive cells was specific as only TGFβ could displace the labelled TGFβ, EGF and TGFα did not influence TGFβ binding (Figure 2).

The results obtained from the radioreceptor assay and the displacement studies demonstrated that a large proportion of the SCLC cell lines examined carried specific high affinity TGFβ receptors. Our results are in part corroborated as one of these cell lines, GLC 19, has previously been reported to be growth inhibited by TGFβ (Lagace et al., 1991). However, in the cited study the cells were not examined for the presence of TGFβ receptors.

To verify that the binding of TGFβ was in fact to the TGFβ receptors, the cell lines were tested by cross-linking. After size fractionation on SDS-PAGE gels, all cell lines found to be TGFβ receptors in the radioreceptor assay also displayed one or more specific bands with calculated Mr = 65,000, 90,000 and 280,000 (Figure 3). These sizes include reduced TGFβ with a Mr of approximately 12,000. The Mr of the corresponding receptors is therefore 53,000, 78,000 and 270,000. The TGFβ receptors have previously been reported as having these calculated molecular weight (Massagué, 1987; Massagué, 1990). This provides further evidence that TGFβ binding was to the TGFβ receptors. One cell line, GLC 16, expressed the type I and II TGFβ receptors in this assay. We could, however, not demonstrate the receptor in the radioreceptor assay, even using a very high protein concentration (>800 μg ml⁻¹). The binding capacity in this cell line could be so low that it was below the detection limit in the radioreceptor assay. In the same cell line, we have found that the receptor was functional in that exogenously added TGFβ acted as a growth inhibitor (Nørgård, P., unpublished observation).

We also examined the expression of TGFβ mRNAs in the panel. In 10/20 SCLC cell lines TGFβ mRNA could be detected (Table I, Figure 4a). In GLC 3 and faintly in DMS 153 an additional band of 1.7 kb was found, the nature of this band is unclear. A mRNA with this approximate size has also been found in male mouse germ cells (Watrin et al., 1991).

We examined 19 of the SCLC cell lines with a probe for TGFβ mRNA (Figure 4b), and in two cell lines (DMS 53 and DMS 114) a single transcript of 3.9 kb was demonstrated. This is in accordance with one of the TGFβ transcripts reported in other human cell lines (Mori et al., 1990), whereas none of the additional TGFβ mRNAs reported (Jakowlew et al., 1991; Mori et al., 1990; Miller et al., 1989b) were detected in the investigated cell lines.

We have previously examined 15 of the cell lines in our panel for expression of phosphorylated retinoblastoma gene product (pRb) (Rygaard et al., 1990). Only the two cell lines (DMS 53 and DMS 114) in which TGFβ mRNA was...
detected, were found to express pRb, and immunocytochemistry demonstrated nuclear localisation of pRb (Rygaard, K., unpublished observation). Other studies (Templeton et al., 1991) have suggested that pRb is functional only when phosphorylated and located in the nucleus. It has recently been reported that the pRb activates the expression of TGFB, (Kim et al., 1992). Provided that the characteristics of pRb in DMS 53 and DMS 114 indicates that the protein is functional, our finding that TGFB, was detected exclusively in the two cell lines also expressing a 'functional' pRb, agrees with the concept that expression of TGFB, is activated by pRb.

Seven cell lines expressed the 3.5 kb TGFB, mRNA (Figure 4c), corresponding to the reported size in other human malignant tissue (Dijke et al., 1988). Two of these cell lines (GLC 14 and 16) also expressed a transcript with a size of approximately 2.5 kb, which is the transcript size of TGFB, mRNA, however, these two cell lines did not express TGFB, mRNA.

The probing for TGFB, and TGFB, was performed with a murine probe, and there may not be perfect homology to the human mRNA. This implies that additional cell lines could be positive following probing with a human probe. Taken together a total of 12 of the 20 examined SCLC cell lines expressed TGFB, mRNA. This finding is in contrast to earlier studies, where a few cell lines have been examined and found to be TGFB, mRNA negative (Söderhäll et al., 1986; Derynk et al., 1987; Lagadec et al., 1991). In one of these studies (Lagadec et al., 1991) the examined cell lines included GLC 14, 16 and 19, PCI H69 and NCI 214, all these were TGFB, mRNA negative. These cell lines were also included in our panel, but we found expression of TGFB, mRNA in GLC 19 and NCI N417. This transcript was detected as a 2.5 kb band using both the full length cDNA and the 267 bp TGFB, fragment. The difference between our results and the previous reported study (Lagadec et al., 1991), is not apparent, but could be due to a difference in sensitivity.

Our results based on a panel of 21 SCLC cell lines have demonstrated that TGFB, receptors were present in seven of 21 SCLC cell lines and more than half of the cells examined expressed TGFB, mRNA. About half of the examined cell lines grew as monolayer cultures and half as floating aggregates, but we could not detect any statistical difference between the growth morphology and the expression of TGFB, or TGFB, mRNAs (Chi-square test with Yates correction and Fisher's exact test, P < 0.2). Coexpression of TGFB, and the ligand was found in six cell lines. These cell lines therefore represent a possible autocrine growth regulation. The question whether the SCLC cell lines produce TGFB protein and if this is biologically active is currently being investigated.

This work was supported by the Danish Cancer Society, the Danish Research Academy, the Hænch's foundation, the Madsen foundation and the Vissing's foundation. The authors thank Mrs C. Jespersen and J. Rørhm for technical assistance.

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