Hydrolysis of transforming growth factor- α by cell-surface peptidases *in vitro*

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Human transforming growth factor- α (h-TGF α), a 50-amino acid residue peptide, was incubated with some purified cell-surface peptidases and with renal microvillar membranes prepared from pig and rat. Hydrolysis was monitored by h.p.l.c. and activity by a biological assay. Prolonged incubation with relatively large amounts of endopeptidase-24.11, aminopeptidase N and peptidyl dipeptidase A (angiotensin-converting enzyme) caused no observable hydrolysis and no detectable loss of biological activity. Incubation with pig renal microvilli also failed to degrade the peptide. In contrast, rat renal microvilli readily degraded h-TGF α , as did endopeptidase-2, which is located in rat renal and intestinal brush borders, but is absent from pig kidneys. This enzyme degraded about 30 nmol of h-TGF α /h per mg of protein. The physiological significance of these results is discussed.

INTRODUCTION

Transforming growth factor- α (TGF α) was first recognized in the culture medium of a retrovirus-transformed fibroblast line (DeLarco & Todaro, 1978) and, later, in that of human tumour cells (Todaro *et al.*, 1980). Other studies have confirmed the role of TGF α in neoplasia (Derynck *et al.*, 1987; Dickson *et al.*, 1987), but the expression of TGF α in embryonic development of mouse and rat tissues, including placenta and kidney (Lee *et al.*, 1985a; Wilcox & Derynck, 1988a; Rappolee *et al.*, 1988), as well as in a wide range of normal cells and tissues, e.g. pituitary cells (Kobrin *et al.*, 1986), brain (Wilcox & Derynck, 1988b), keratinocytes (Coffey *et al.*, 1987), activated macrophages (Madtes *et al.*, 1988) and mammary epithelium (Liu *et al.*, 1987; Smith *et al.*, 1989), indicates a probable role of TGF α during normal development. Other reported roles are in wound healing (Schultz *et al.*, 1987), angiogenesis (Schreiber *et al.*, 1986) and bone resorption (Ibbotson *et al.*, 1983; Stern *et al.*, 1985). Recently, TGF α has also been reported in normal human plasma (Kato *et al.*, 1990).

The sequences of purified forms of TGF α from rodent and human tumour cells reveal about 30% identity with epidermal growth factor (EGF), particularly in the conservation of six cysteine residues (Massague, 1983; Marquardt *et al.*, 1983). The similarity in conformational structure between TGF α and EGF would explain their interaction with the EGF receptor (Marquardt *et al.*, 1984). There is also a high degree of homology among species, with human TGF α (h-TGF α) a 50-amino-acid-residue polypeptide (Fig. 1) differing from rat TGF α by only four residues. However, cloning of cDNA has revealed the existence of some larger precursor forms (pro-TGF α), containing 160 residues in the human (Derynck *et al.*, 1984) and 159 residues in the rat (Lee *et al.*, 1985b). These precursors possess three additional domains: about 50 residues at the N-terminus, a transmembrane hydrophobic domain and a 35-residue cytoplasmic domain, rich in cysteine residues, several of which are palmitoylated (Brigman *et al.*, 1987; Gentry *et al.*, 1987; Teixido *et al.*, 1987). The mature TGF α sequence is flanked by valine- and alanine-rich sequences and the processing and release of TGF α is dependent on two cleavage events at the C- and N-termini involving Ala-Val peptide bonds (Teixido *et al.*, 1990) by extracellular or cell-surface proteinases as yet undefined.

The subsequent metabolism of the secreted TGF α appears to be unknown, and this has prompted us to examine the susceptibility of the molecule to attack by some of the widely distributed cell-surface peptidases. One of these, endopeptidase-24.11 (EC 3.4.24.11), has a large repertoire of susceptible peptides, including tachykinins, enkephalins, bradykinin, angiotensins, and brain and atrial natriuretic peptides (Matsas *et al.*, 1984; Stephenson & Kenny, 1987a,b; Bourne & Kenny, 1990). It is not a proteinase, but it does hydrolyse interleukin 1 β (Pierart *et al.*, 1988), although not interleukin 1 α or tumour necrosis factor α (Y. Choudry & A. J. Kenny, unpublished work). The primary specificity of this enzyme is for bonds involving the α -amino function of hydrophobic residues and there are about a dozen such potential sites in TGF α . However, other structural constraints crucially affect the efficiency of hydrolysis, including subsite binding and conformation (Matsas *et al.*, 1984). For example, the hydrolysis within the six-membered rings of oxytocin and vasopressin is extremely slow (Stephenson & Kenny, 1987a) while for the nine-residue ring of endothelin and the 17-residue ring of the natriuretic peptides hydrolysis is rapid (Vijayaraghavan *et al.*, 1990; Stephenson & Kenny, 1987b; Bourne & Kenny, 1990). The ring sizes of h-TGF α might therefore offer susceptible points of attack provided other criteria were satisfied. A second cell-surface endopeptidase (endopeptidase-2, E-2, EC 3.4.24.-) isolated from rat kidneys has a more restricted tissue distribution and is less efficient in hydrolysing neuropeptides but it does exhibit greater potential as a proteinase (Kenny & Ingram, 1987; Stephenson & Kenny, 1988; Barnes *et al.*, 1989). The head and tail peptide chains might also be susceptible to hydrolysis by exopeptidases, such as amino-

Fig. 1. Structure of h-TGF α

The 50-residue form of the peptide is shown (Derynck *et al.*, 1984).

peptidase N (EC 3.4.11.2, AP-N) and peptidyl dipeptidase A (EC 3.4.15.1, angiotensin-converting enzyme), which respectively remove single amino acids from the *N*-terminus or dipeptides from the *C*-terminus [for review of this group of enzymes, see Kenny *et al.*, (1987)].

In the present paper we report the results of experiments in which h-TGF α has been treated with these purified peptidases, any degradation being monitored by h.p.l.c. analyses and by biological assay.

EXPERIMENTAL

Materials

Recombinant h-TGF α . Recombinant h-TGF α was supplied by Dr. H. Gregory (ICI Plc) and characterized as described by Gregory *et al.* (1988).

Peptidases. Endopeptidase-24.11, aminopeptidase N and peptidyl dipeptidase A (angiotensin-converting enzyme) were purified from pig kidneys as noted (Stephenson & Kenny, 1987a) and endopeptidase-2 was isolated from rat kidneys (Kenny & Ingram, 1987). Peptidase inhibitors were obtained from Sigma Chemical Co.

Methods

Incubations of TGF α with peptidases and membranes. The incubation mixture (volume 100 μ l) contained 10 μ g of h-TGF α , 10 μ g of purified peptidase and 20 mM-Tris/HCl, pH 7.4. When inhibitors were present, the concentrations were: 1 μ M-phosphoramidon, 1 μ M-amastatin, 0.1 mM-di-isopropyl fluorophosphate, 1 μ M-enalaprilat, 10 μ M-actinonin; all were preincubated (37 °C) for 15 min with the peptidase before addition of the peptide. h-TGF α was incubated at 37 °C for various times up to 18 h. Renal microvillar membranes were prepared from pig and rat kidneys (Booth & Kenny, 1974). Before use they were resuspended in 10 ml of 50 mM-sodium phosphate/150 mM-NaCl, pH 7.4, and pelleted at 30000 *g* for 30 min. The pellet was taken up in incubation buffer (10 mg of protein/ml); 0.1 mg was used in the incubations with 10 μ g of h-TGF α . Incubations were terminated by heating to 95 °C for 10 min and removal of any pellet by centrifugation in a Microfuge.

Reduction and carboxymethylation of h-TGF α . To 10 μ g of h-TGF α (2 nmol) in 100 μ l of incubation mixture was added 8 μ l of 0.5 M-dithiothreitol in 0.3 M-Tris/HCl, pH 8.6, and the mixture incubated at 37 °C for 1 h. Iodoacetic acid (20 μ l of 0.5 M in 0.3 M-Tris/HCl, freshly made) was then added and incubated at 37 °C for 1 h. All incubations were carried out under N₂.

H.p.l.c. analysis of h-TGF α . A Waters C₁₈ μ Bondapak column was used, the elution being achieved in 20 min by a gradient of 4–45 % acetonitrile in 0.05 % trifluoroacetic acid at a flow rate of 2 ml/min.

Biological assay of h-TGF α . The fraction corresponding to TGF α peak (retention time on h.p.l.c., 15 min) was collected and freeze-dried. TGF α was bioassayed by measuring the incorporation of tritium-labelled thymidine in response to this mitogen in NIH 3T3 cells. The methodology employed was essentially as described in Gregory *et al.* (1988) but gentamicin was omitted from the medium and cells were grown in an atmosphere containing 10 % CO₂.

RESULTS

H.p.l.c. of h-TGF α

The sample of recombinant h-TGF α was homogeneous by h.p.l.c., producing a sharp peak (retention time 15 min, Fig. 2a).

This peak was abolished when the peptide was reduced and carboxymethylated, being replaced by a new peak of equal area (retention time 16.7 min, Fig. 2b). The reagents produced peaks eluted in the early part of the gradient (5–8.5 min), well removed from the peaks of the native and extended forms of h-TGF α .

Incubation of h-TGF α with endopeptidase-24.11

Incubations for periods of 0, 2, 4 and 18 h without inhibitors and 0 and 18 h with 1 μ M-phosphoramidon yielded identical h.p.l.c. profiles (e.g. Fig. 2c). When the samples were subjected to reduction and carboxymethylation, to exclude the possibility that limited hydrolysis occurred within one of the loops of the peptide without affecting the retention time of the unreduced molecule, no evidence was obtained for the hydrolysis of any peptide bonds (e.g. Fig. 2d).

Incubations of h-TGF α with peptidyl dipeptidase A and aminopeptidase N

The existence of seven-residue *N*- and *C*-terminal extensions in h-TGF α raised the possibility that these two exopeptidases might inactivate the factor by a sequential attack at the extremities. Incubations for periods of 0, 4 and 18 h with and without the inhibitors of peptidyl dipeptidase A (1 μ M-enalaprilat) and aminopeptidase N (1 μ M-amastatin) all yielded identical h.p.l.c. profiles (results not shown).

Incubation of h-TGF α with endopeptidase-2

Incubations were carried out for periods of 0, 1, 2, 4, 8 and 18 h, each in the presence of a cocktail of inhibitors, including amastatin, enalaprilat, di-isopropyl fluorophosphate and phosphoramidon, to block any contaminating peptidases

Fig. 2. H.p.l.c. profiles of h-TGF α

See the Experimental section for details. (a) h-TGF α (retention time 14.96 min); (b) as (a) after reduction and carboxymethylation (retention time 16.6 min); the four peaks at 4.8, 5.3, 6.3 and 8.4 min are attributable to the reagents used; (c) h-TGF α after 18 h incubation with endopeptidase-24.11; (d) as (c), after reduction and carboxymethylation; no peptide products are observed in either incubation.

Fig. 3. Effect of membrane peptidases on h-TGF α

See the Experimental section for details. The peak area of h-TGF α (retention time 15 min) has been plotted on a log scale as a percentage of the zero-time value. ■, Endopeptidase-24.11; □, peptidyl dipeptidase A; ▲, aminopeptidase N; ●, endopeptidase-2; △, microvillar membranes (pig); ○, microvillar membranes (rat). After 18 h incubation with endopeptidase-2, no h-TGF α peak was detected.

(Stephenson & Kenny, 1987a). In another incubation for 18 h, these four inhibitors and 10 μ M-actinonin were present. H.p.l.c. analyses showed a decrease in h-TGF α peak area with only 50 % remaining at 4 h and total disappearance at 18 h (Fig. 3). In the presence of actinonin no hydrolysis was observed (result not shown).

Incubation of h-TGF α with renal microvillar membranes of pig and rat kidneys

Incubation of h-TGF α with pig renal microvillar membranes for up to 8 h revealed no change in the h-TGF α peak area (Fig. 3), and the presence of either 10 μ M-actinonin or the cocktail of four inhibitors at 0 and 8 h had no effect. However, rat renal microvillar membranes degraded h-TGF α to 50 % in 8 h in the presence of the four inhibitors (Fig. 3), but 94 % inhibition of hydrolysis was observed in the presence of 10 μ M-actinonin (result not shown). In the incubations with rat membranes, two new peaks were generated, being detected at 2 h and increasing with time to 8 h. These products had retention times of 5.1 and 8.3 min, and their total areas approximated to the loss of area of the h-TGF α peak. Actinonin caused 77 % and 51 % inhibition of the production of these two products.

Biological assay of h-TGF α after incubation with endopeptidase-24.11, peptidyl dipeptidase A and aminopeptidase N

Although no indication of hydrolysis by these three peptidases was obtained by h.p.l.c. analyses, we could not exclude the possibility that inactive products were generated, but not resolved

Fig. 4. Biological assay of h-TGF α after incubation with endopeptidase-24.11, peptidyl dipeptidase A and aminopeptidase N

See the Experimental section for details. Zero-time controls (mean of two experiments) are shown by a dashed line. 18 h incubations: ○, control (no peptidases); ●, endopeptidase-24.11; ■, peptidyl dipeptidase A; ▲, aminopeptidase N.

from the substrate by h.p.l.c. in the conditions used. The fraction corresponding to the h-TGF α peak was collected from a series of incubations, freeze-dried and each subjected to biological assay. The samples assayed were: two zero-time standards, a control incubated for 18 h at 37 °C in the absence of peptidases and the 18 h samples after incubation with endopeptidase-24.11, peptidyl dipeptidase A and aminopeptidase N. The recovery of activity in the zero-time standards (dashed line in Fig. 4) was at the predicted level, showing that no significant loss had occurred in isolating the peptide in the peak fraction. The samples incubated for 18 h showed slight loss of activity compared with the zero-time standards. However, the assay curves of the incubated controls and peptidase incubations were essentially superimposable showing that none of these three peptidases inactivated h-TGF α in these conditions.

DISCUSSION

The only membrane enzyme which degraded h-TGF α in these experiments was endopeptidase-2, half of the peptide disappearing from the peak in 3 h, a rate corresponding to about 30 nmol/h per mg of enzyme over this period. Hydrolysis by rat renal microvillar membranes was also observed, and this activity was not inhibitable by phosphoramidon, amastatin, di-isopropyl fluorophosphate or enalaprilat. In contrast, 10 μ M-actinonin inhibited strongly, a result consistent with a contribution by endopeptidase-2 to the hydrolysis of TGF α by the rat membranes. The bidentate compound, actinonin (Umezawa *et al.*, 1985), is a general inhibitor of many metallopeptidases, but endopeptidase-2 is especially sensitive. The IC₅₀ value for endopeptidase-2 in an assay using ¹²⁵I-labelled insulin B chain as substrate is 35 nM compared with 9.4 μ M for endopeptidase-24.11 and 330 μ M for peptidyl dipeptidase A (J. Ingram & A. J. Kenny, unpublished work). Furthermore, incubation with pig renal microvillar

membranes did not lead to any observable hydrolysis of h-TGF α . One difference in the complement of peptidases between the two membranes is the presence of endopeptidase-2 in the rat (Kenny & Ingram, 1987). The physiological significance of the hydrolysis of TGF α by purified endopeptidase-2 is doubtful: endopeptidase-2 has a very limited tissue distribution in the rat, being abundant in renal and intestinal brush borders, but not immunologically detectable in most other tissues save for weak staining in salivary glands and thyroid (Barnes *et al.*, 1989).

Endopeptidase-24.11 is, in contrast, a widely distributed cell-surface peptidase with a large repertoire of susceptible peptide substrates (Kenny *et al.*, 1987). These studies show, beyond doubt, that h-TGF α is not among its likely targets. The incubation conditions used equal amounts (by weight) of enzyme and peptide and extended incubation times to 18 h. No peptide products were observed and biological assay showed no loss of activity. The two exopeptidases tested, aminopeptidase N and peptidyl dipeptidase A, were also unable to inactivate TGF α . These two ectoenzymes are also widely distributed on many cell surfaces, especially on the vascular endothelium. The metabolic fate of h-TGF α therefore remains to be elucidated. These experiments performed *in vitro* lend no support for cell surface peptidases playing a significant role, unless an enzyme resembling endopeptidase-2 in its activity but with a much wider distribution remains to be discovered. It is more likely that h-TGF α is degraded *in vivo* by lysosomal peptidases after internalization, as seems probable in the case of interleukin 1 α (Poole *et al.*, 1990).

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