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β-TRANSFORMING GROWTH FACTOR IS STORED IN HUMAN BLOOD PLATELETS AS A LATENT HIGH MOLECULAR WEIGHT COMPLEX

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Human blood platelets, the richest known source of β-Transforming Growth Factor extractable under acid conditions, release in neutral extracts (pH 7.2) a latent form of this growth factor with an apparent molecular weight of 400 Kd. This latent form, poorly active on rat NRK-49F indicator cells in soft agar assays can be activated by exposure to acid pH or 8 molar urea. The acid activated β-Transforming Growth Factor from neutral extracts elutes on Biogel P60, in 1 molar acetic acid, as a broad peak of apparent molecular weight 15-30 Kd, like when this factor is extracted from platelets by the usual acid-ethanol procedure. Moreover, β-Transforming Growth Factor from both acid activated neutral extracts and from acid-ethanol extracts elutes on reverse phase at 30 % acetonitrile. We suggest that β-Transforming Growth Factor is stored in human blood platelets as a poorly active high molecular weight complex which may be dissociated and activated in appropriate in vivo microenvironments.

β-Transforming Growth Factor (β-TGF) is a cell produced factor (1,2) which may either stimulate or inhibit the growth of attached or unanchored cells depending on the origin of these cells and the presence of other growth factor(s), such as epidermal growth factor (EGF) or platelet derived growth factor (PDGF) (3,4,5,6). It is composed of two homodimers of 112 aminoacids each (7), linked by disulfide bridges and probably synthetized as precursors of 391 aminoacids. β-TGF is present in the conditioned medium of most cell cultures as a high apparent molecular weight complex which is only poorly effective in promoting the anchorage-independant growth of sensitive fibroblastic target cells, such as rat NRK-49F cells in the presence of EGF, or mouse AKR-2B cells (8,9). This latent form of β-TGF can be converted into an active low apparent molecular weight (16 kd) component in acid conditions (10).

ABBREVIATIONS:

β-TGF, β-Transforming Growth Factor ; EGF, epidermal growth factor ; PBS, phosphate buffered saline ; HCl/BSA, 4 mM hydrochloric acid-0.5 mg/ml bovine serum albumin ; ED 50 %, efficient dose 50 % ; TFA, trifluoroacetic acid.

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Human blood platelets are the richest known source of β-TGF (11), which raises the possibility that β-TGF is released by thrombocytes in the process of contusion and blood extravasation and stimulates wound healing (12). Here we relate that β-TGF is stored in platelets as an essentially inactive, high apparent molecular weight complex and is activable by exposure to acid pH or urea.

MATERIALS AND METHODS

Platelet extracts.

Standard platelet concentrates (2-5 days old) were obtained through the courtesy of the Centre National de Transfusion Sanguine, Courtaboeuf, Les Ulis, France, and centrifuged (600 g ; 10 min ; 5°C) to remove the majority of red blood cells. The platelets were washed with Phosphate Buffered Saline (PBS) and centrifuged (3000 g ; 10 min ; 5°C). Washed platelets (1 gm wet weight) were added to 20 ml of PBS, 1 mM PMSF (phenylmethylsulfonylfluoride) and were sonicated (30 seconds, setting 8 on a 100 watt apparatus Medical Scientific Equipment), then allowed to stand at 4°C for 2 hrs. Cell debris were removed by centrifugation (100.000 x g for 1 hr). The supernatant was considered as the neutral extract. Aliquots of the neutral extract were acidified by dialysis against 1 M acetic acid, followed by lyophilisation and resolubilisation in 4 mM HCl plus 0,5 mg of bovine serum albumin per ml (HCl/BSA). It should be noted that this resolubilisation was done by diluting at least 10 fold the residue in HCl/BSA, in order to avoid the loss of material. Other aliquots of the neutral extract were brought to 8 M urea and after 2 hrs at room temperature, extensively dialysed against PBS to remove urea. Acid/ethanol extract was prepared according to Assoian et al (11) from 1 gm (wet weight) of platelets. The amount of protein in the extract was determined using the Bradford Biorad Protein Assay.

Soft agar growth assay.

β-TGF activity was measured (8) by the capacity of serial 2 fold dilutions to induce the formation of anchorage independant colonies in agar-gelified medium by 3000 NRK-49F cells per 1.9 cm^2 well, in the presence of 5 ng of EGF per ml. Colonies were counted under the microscope at ten days. One unit of β-TGF activity is defined as that biological response resulting in 50 % of maximal colony formation (> 50 cells) in the presence of EGF (5 ng/ml) corresponding to the ED_{50}. The maximal response of the assay was about 700 colonies/well (23 % cloning efficiency).

Chromatographic procedures.

Biogel P60. An aliquot of the neutral extract was layered on a Biogel P60 column (1.6 x 40 cm) and eluted with PBS (flow rate 14 ml/hr, fraction volume 2.2 ml). Aliquots (1.0 ml) of each fraction were acidified by dialysis (in Spectropor 3 membranes) against 1 M acetic acid, freeze dried and redissolved in HCl/BSA. The acidified and neutral aliquots of each fraction were assayed in parallel for β-TGF activity. Gel filtration of β-TGF-containing preparations under acidic conditions was performed similarly but using 1 M acetic acid as eluant.

Superose 12. Protein from the neutral extract was applied to a Superose 12 column (type HR x 300, system FPLC, Pharmacia) and eluted with PBS. Protein from the acid-ethanol extract was lyophilised and dissolved in 0.1 M ammonium acetate, pH 5.0, before application to the column and eluted with this same buffer. In each case the flow rate was 0.2 ml/min and 0.4 ml fractions were collected. The fractions of the neutral extract were acidified as above and the fractions of both extracts were lyophilised and redissolved in HCl/BSA.

Reverse Phase-FPLC. The 15-30 Kd peaks of β-TGF obtained by Biogel P60 gel filtration, using 1 M acetic acid as eluant, of acid activated neutral extract or of acid-ethanol extract were subjected to reverse phase chromatography on a Pro RPC 5/2 column, bed size 5 x 25 mm (Pharmacia) equilibrated with 25 % acetonitrile-water containing 0.1 % TFA. The β-TGF containing material was lyophilised, reconstituted in 0.1 % TFA in water and injected on the column. After a 12 minute elution with 25 % acetonitrile-water-0.1 % TFA, a 25 %-50 % acetonitrile-water-0.1 % TFA gradient was passed during 32 minutes. The flow rate was 0.8 ml/min and 0.8 ml fractions were collected. Aliquots (200 µl) of these fractions were lyophilised, dissolved in 500 µl of HCl/BSA and 10 µl volumes assayed for β-TGF activity.

RESULTS AND DISCUSSION

First, using the same batch of human blood platelets, we compared the β-TGF activity on NRK-49F cells of acid-ethanol extracts prepared according to Assoian et al. (11) and of neutral extracts prepared by sonication of the platelets in PBS at pH 7.2. As shown in Table 1, the acid extract was about 300 fold more active in inducing anchorage independant growth of NRK-49F cells in the presence of EGF than the neutral extract. The activity of the latter extract could be enhanced 300 fold by acidification, i.e., dialysis against 1 M acetic acid, followed by lyophilisation and

TABLE I β-TGF activity in acid ethanol extract or in neutral extract of human platelets and in neutral extract activated by acidification or 8 M urea

Nature of the extract	Protein extracted per gr of platelets	β-TGF activity in ED 50 % unit per ug of protein	Total β-TGF activity present in 1 gr of platelets
Acid ethanol extract	24 mg	13.8	331200
Neutral extract	120 mg	0,01	1200
- After acidification	120 mg	3.12	375000
- After urea treatment	120 mg	2.2	264000

It should be noted that the acid-ethanol extraction of the cell debris removed by the 100.000 x g centrifugation following preparation of the neutral extract yielded about 1/5 of the total β-TGF activity present in the acid activated neutral extract. About the same amount of β-TGF was also extractable from the pellet of the acid ethanol extraction.

resolubilizing in 4 mM HCl containing 0.5 mg/ml of bovine albumin. Activation was also observed following treatment with 8 M urea. Dilution of the activated extract in the neutral extract did not reduce the activity of the former extract more than its dilution in PBS (not shown), which suggests that the activation is not due to the destruction of an inhibitor present in excess in the neutral extract and able to associate in vitro with the active β-TGF. Moreover, the anchorage-independant growth-inducing activity monitored by NRK-49F cells was strictly dependant on the presence of EGF.

Next, we compared the apparent molecular weight of the activable, latent β-TGF present in the neutral extract to that of the active β-TGF present in the neutral extract activated by acidification. Gel filtration of the neutral extract on a Biogel P60 column with PBS as eluant showed that the latent β-TGF activity revealed by subsequent acidification of each fraction was solely in the exclusion volume, without any activity corresponding to low apparent molecular weight components (Fig. 1A). In contrast, the β-TGF of the neutral extract activated by acidification prior to gel filtration was present, when eluted with 1 M acetic acid in a single broad peak corresponding to an apparent molecular weight of 15-30 kd (Fig. 1B). Furthermore, a second filtration on the same column with 1 M acetic acid as eluant of the excluded fractions containing the latent β-TGF activity again revealed a broad 15-30 kd peak, but broken in the centre (Fig. 1C). Confirmatory results (Fig. 2) were obtained by FPLC (Pharmacia) using a Superose 12 column. Latent β-TGF from the neutral extracts eluted in PBS with an apparent molecular weight of 400 kd, whereas the β-TGF activated by acidification in neutral extracts and the active β-TGF from acid-ethanol extracts eluted with an apparent molecular weight of 12 kd in 0.1 M ammonium acetate (pH 5). We should note here that 1 M acetic acid does not permit elution of proteins on Superose columns.

In order to further characterize the β-TGF activity of the acid activated neutral extracts and of the acid ethanol extracts, each extract was first filtered on a Biogel P60 column in acid conditions, and the active fractions, corresponding to an apparent molecular weight of 15-30 kd were then pooled and rechromatographed on a reverse phase column (Pro RPC 5/2, Pharmacia). Both activities eluted on an

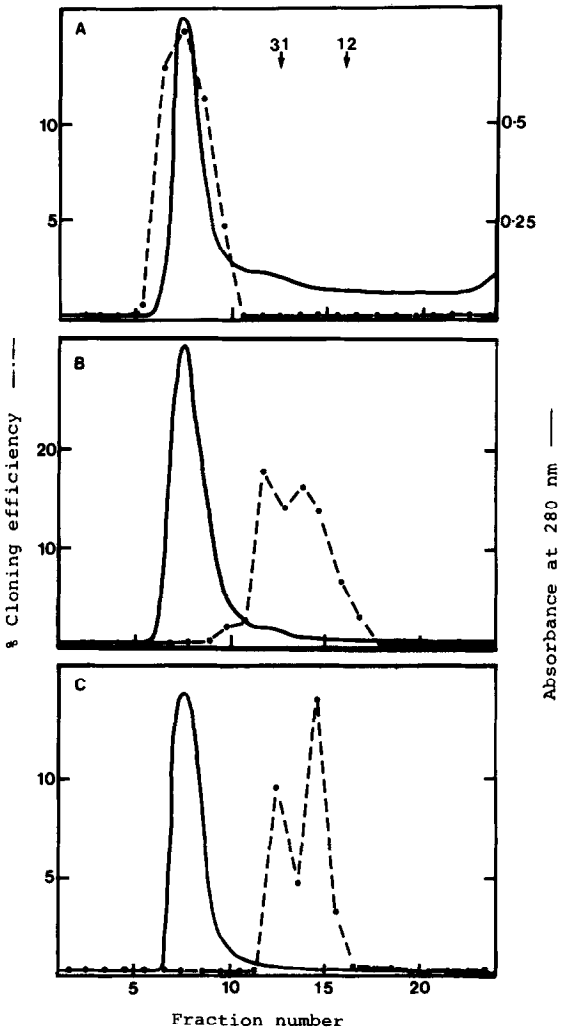

FIGURE 1 : Biogel P60 chromatography of platelet extract.

Protein profile as determined by absorbance at 280 nm (—). Vertical arrows show from left to right, the elution positions of carbonic anhydrase (Mr : 31000) and cytochrome C (Mr : 12000) respectively. Two other markers, bovine serum albumin (Mr : 68000 ; Vo) and vitamin B12 (Mr : 1300) were used but are not shown to avoid cluttering the diagram. Elution position of platelet derived β -TGF determined by percent cloning efficiency of NRK-49F cells induced in the presence of EGF (5 ng/ml) by aliquots of column fractions (—●—).

A/ Soluble material (35 mg of protein) from the neutral platelet extract was layered on a Biogel P60 column (1.6 cm x 40 cm) and eluted with PBS at a flow rate of 14 ml/hr. Fractions of 2,2 ml were collected and assayed for protein content. Aliquots (1 ml) from the fractions were dialysed against 1 M acetic acid and freeze dried. The residues were sterilised with ^{60}Co γ -irradiation and dissolved in sterile HCl/BSA (1 ml). β-TGF activity was measured in aliquots (200 µl) from neutral fractions and 5-10 µl aliquots of acidified material. The neutral fractions were all inactive except for a very low activity in the excluded volume (not shown). The excluded fractions after activation had a specific activity about 40 fold higher than before activation (data not shown).

B/ Thirty five mg of protein from the neutral extract were dialysed against 1 M acetic acid and chromatographed on Biogel P60 which had been equilibrated in 1 M acetic acid. Fractions containing 2,2 ml were collected at a flow rate of 14 ml/hr. Aliquots (500 µl) were lyophilized and residues dissolved in HCl/BSA (1 ml) and tested as above.

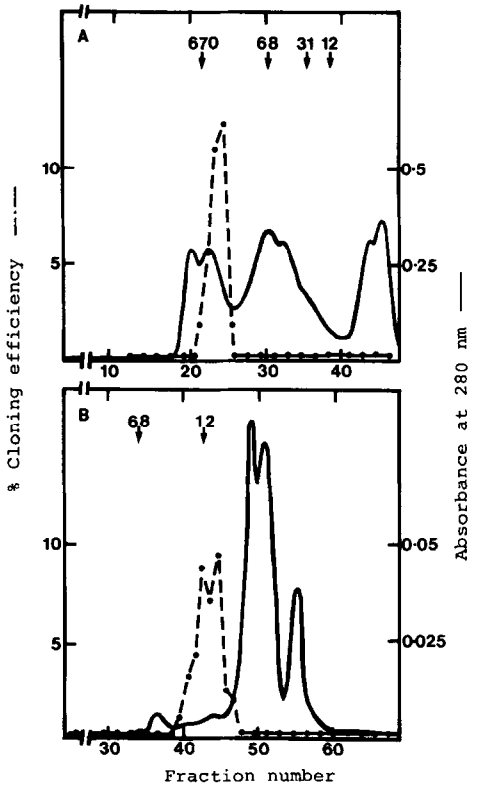

FIGURE 2 : FPLC of platelet-derived β-TGF on Superose 12.

Protein profile as determined by absorbance at 280 nm (——). Vertical arrows show, from left to right, the elution positions of thyroglobulin (Mr : 670000) ; bovine serum albumin (Mr : 68000) ; carbonic anhydrase (Mr : 31000) and cytochrome C (Mr : 12000) respectively.

A/ Protein (770 µg) from the neutral extract were applied to a Superose 12 column (HR 10 x 300 mm, Pharmacia), which had been equilibrated in PBS. The flow rate was 0,2 ml/min and 0,4 ml fractions were collected. Each fraction was dialysed against 1 M acetic acid, freeze dried and resolubilized in HCl/BSA (1 ml). Aliquots (5 µl) from these fractions were assayed as above for β-TGF activity.

B/ Acid ethanol extract was prepared according to Assoian et al. (11) from 1 g (wet weight) of platelets. The solubilized platelet extract, 2 ml in 1 M acetic acid, was lyophilised and residues were solubilized in 0,1 M ammonium acetate pH 5. An aliquot of 520 µg of protein was applied to a Superose 12 column which had been equilibrated in the sample solvant (0,1 M ammonium acetate pH 5). The flow rate was 0,2 ml/min and 0,4 ml fractions were collected. Each fraction was freeze dried and resolubilized in HCl/BSA (500 ul). Aliquots of 100 µl volume from these fractions were tested as above for β-TGF activity.

acetonitrile gradient at 30 % acetonitrile, suggesting that the active component in the two extracts is the same (Fig. 3).

Our results thus show that blood platelets contain β-TGF in the form of a high apparent molecular weight complex with low anchorage independant growth

C/ Three mg of protein from the excluded neutral fraction of a Biogel P60 run were pooled, dialysed against 1 M acetic acid and rechromatographed exactly as described in B/. Aliquots (200 µl) were freeze dried and residues were resolubilized in HCl/BSA (500 µl) and 5-10 µl of each fraction were assayed for β-TGF activity.

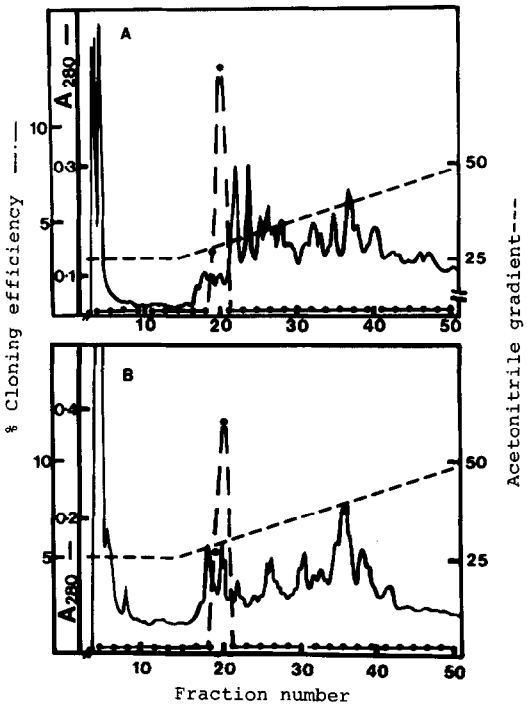

FIGURE 3 : Chromatography of β-TGF on reverse phase Pro RPC 5/2 (Pharmacia).

Protein profile as determined by absorbance at 280 nm (——).

A/ Neutral extract : The neutral extract was acidified and chromatographed on Biogel P60 as shown in Figure 1 panel B. After lyophilization, the active material was reconstituted in 2 ml of 0,1 % trifluoroacetic acid in water. Aliquots containing 1,2 mg of protein were injected on a Pro RPC 5/2 column (bed size : 5 x 25 mm) previously equilibrated with 25 % acetonitrile-water containing 0,1 % TFA. Elution was at room temperature and at a flow rate of 0,8 ml/min. After a 12 min elution with 25 % acetonitrile-water-0,1 % TFA to remove unbound protein, a 25-50 % acetonitrile-water-0,1 % TFA gradient was passed during 32 min. Fractions of 0,8 ml were collected. Aliquots (200 µl) of these fractions were lyophilized, resolubilized in HCl/BSA (500 µl) and 10 µl volumes were assayed for β-TGF activity as above. In the absence of EGF, no β-TGF activity was observed. Another aliquot (200 µl) was tested for protein content.

B/ Acid-ethanol extract : Acid ethanol extract was first chromatographed on Biogel P60 with 1 M acetic acid. The β-TGF activity corresponding to 15-30 kd was pooled and subjected to reverse phase chromatography as described above.

promoting activity towards NRK-49F cells and that acidification dissociates this complex and reveals the active β-TGF of 15-20 kd apparent molecular weight. As urea also activates latent β-TGF, it is unlikely that activation results from the rupture of a peptide linkage. Rather β-TGF might be associated with a binding protein(s), perhaps involving hydrogen bonding, which causes the loss, or a reduction, of its in vitro biological activity. The question remains open as to whether the existence of such a complex is of biological interest. One can speculate that the release of the inactive complex in the vicinity of wounded tissues may be followed by

its activation by cell products, perhaps enzymes, and/or its presence in an acidic micro-environment. Such an activation may constitute a regulatory step. But one can also consider that activation is simply an in vitro characteristic, linked to the use of special target cells such as NRK-49F or AKR-2B cells. However, as latent β-TGF is fully active without prior acidification when its anchorage-independant growth promoting capacity is tested on tumorigenic cells (NRK-4 clone) or on chicken or rat cells sensitized by viral oncogenes (13,14), it is possible that non-neoplastic cells, such as NRK-49F or AKR-2B cells, distinguish between latent and activated β-TGF, whereas neoplastic cells respond equally well to both.

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