

Transforming growth factors $\beta 1$ and $\beta 2$ as well as milk growth factor decrease anti-CD3-induced proliferation of human lymphocytes without inhibiting the anti-CD3-mediated increase of $[Ca^{2+}]_i$ and the activation of protein kinase C

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Received 7 April 1989

Porcine transforming growth factor 1 and 2 (pTGF- $\beta 1$ and - $\beta 2$) and milk growth factor (MGF) at 1 ng/ml significantly inhibited the proliferation of human lymphocytes induced by anti-CD3 antibodies. In contrast, the anti-CD3-mediated increase of intracellular Ca^{2+} and the activation and translocation of protein kinase C were not affected by the transforming growth factors.

Ca^{2+} ; Protein kinase C; Transforming growth factor β

1. INTRODUCTION

Among the earliest events detectable in human lymphocyte stimulation (review [1]) are an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) and the activation and translocation of a Ca^{2+} /phospholipid-dependent protein kinase (protein kinase C, PK-C) both of which can be induced by anti-CD3 antibodies [2,3]. The transforming growth factors β (TGF- β) (review [4]) are thought to play an important role in the control of cellular proliferation and have been shown to inhibit lymphocyte stimulation. We investigated whether TGF- $\beta 1$, - $\beta 2$ and milk growth factor, a bovine TGF- $\beta 2$ -like molecule, interfered with the increase of $[Ca^{2+}]_i$ and the activation of PK-C in human anti-CD3-stimulated lymphocytes.

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2. MATERIALS AND METHODS

2.1. Chemicals

Medium HB 102 (DuPont, Dreieich) was supplemented with 2 mM glutamine, 100 U/100 μ g/ml penicillin/streptomycin, 10 mM HEPES, 1 mM pyruvate and the HB 102 serum supplement. The mAbs BMA 030 and BMA 031, which recognize the human CD3 structure or the human T cell receptor (TCR) respectively, were obtained from Behringwerke (Marburg). Porcine transforming growth factor $\beta 1$ and $\beta 2$ (pTGF- $\beta 1$ - and - $\beta 2$) were obtained from R&D Systems (Minneapolis, MN). Milk growth factor (MGF) was kindly donated by Ciba-Geigy and is a 25 kDa dimer from bovine milk purified to homogeneity, which together with EGF induces anchorage-independent growth in 49F fibroblasts and is indistinguishable from bovine TGF- $\beta 2$. (Cox, D. et al., in preparation).

2.2. Cell culture

PBL from buffy coats were obtained from the local transfusion centre and washed twice.

2.3. [³H]Thymidine incorporation

PBL (1×10^6 /ml) were cultured in HB 102 in 96-well microtiter plates in 4-fold replicates and stimulated with 20 ng/ml BMA 030, TGF- $\beta 1$, - $\beta 2$ or MGF (10-0.001 ng/ml) was

Table 1
Effect of pTGF- β 1, pTGF- β 2 and MGF on anti-CD3-induced [3 H]thymidine incorporation in human lymphocytes

	Experiment					
	1		2		3	
\emptyset	450 ^a \pm 73		241 \pm 141		234 \pm 32	
+ BMA 030	14 140 \pm 825		53 778 \pm 869		36 589 \pm 2812	
	pTGF- β 1		pTGF- β 2		MGF	
+ 5 ng/ml	9 578 \pm 945		31 255 \pm 2 453		23 905 \pm 1 843 ^b	
+ 1 ng/ml	7 204 \pm 445		37 735 \pm 2 990		23 702 \pm 1 049	
+ 0.1 ng/ml	11 093 \pm 445		48 746 \pm 835		34 163 \pm 1 555	
+ 0.01 ng/ml	13 914 \pm 899		48 589 \pm 2 697		37 804 \pm 2 039	
+ 0.001 ng/ml	14 287 \pm 1 049		53 195 \pm 3 009		40 181 \pm 1 132	

^a Values: cpm

^b 10 ng/ml MGF

Human PBL (3 different donors) were stimulated for 72 h with 20 ng/ml of the anti-CD3 mAb BMA 030 with or without pTGF- β 1, - β 2 or MGF. [3 H]Thymidine was added for the last 4 h of culture

added at the start of the culture. Cultures were pulsed with [3 H]thymidine (0.5 μ Ci/well, spec. act. 5 Ci/mmol) during the last 4 h of a 72 h culture period.

2.4. Measurement of PK-C translocation

Cells (5×10^6 /ml) were cultured in RPMI 1640 (supplemented with 20 mM Hepes and 5 mg/ml defatted bovine serum albumin) at 37°C with or without 1 ng/ml TGF- β 1 or - β 2 for at least 45 min before adding 20 ng/ml BMA 030. 15 min after the addition of the mAb the cells were spun down and the membrane-bound and cytosolic fractions of PK-C were prepared as described [3].

2.5. Measurement of cytosolic free calcium levels [Ca^{2+}]_i

Cells (1×10^6 /ml) were preincubated for at least 45 min with 1 ng/ml pTGF- β 1 or - β 2. [Ca^{2+}]_i was measured with fura-2 [5]. During all subsequent steps of washing, loading with fura-2 and actual measurement, the concentration of TGF- β was kept constant at 1 ng/ml. Cells were washed twice with PBS and incubated at a density of 10^6 cells/ml at 37°C for 30 min in the presence of 5 μ M fura-2/AM. The labelled cells were washed in PBS and resuspended in PBS supplemented with 1 mM CaCl₂, 1 mM MgCl₂ and 1 g/l glucose. Aliquots of 1.5×10^6 cells in 1.5 ml were placed in a fluorometer (Aminco SPF-500) in magnetically stirred cuvettes, the temperature being maintained

Table 2
Effect of TGF- β 1, TGF- β 2 and MGF on anti-CD3-mediated activation of protein kinase C in human PBL

	Expt 1		Expt 2		Expt 3	
	Cytosol	Membrane	Cytosol	Membrane	Cytosol	Membrane
\emptyset	1488	397	1012	225	1143	161
TGF/ MGF	1532	313	1128	257	1178	205
BMA 030	682	933	705	726	524	630
BMA 030 + TGF/ MGF	557	940	762	807	481	617

Human PBL were stimulated with 20 ng/ml of the anti-CD3 mAb BMA 030 with or without 1 ng/ml pTGF- β 1 (expt 1), in pTGF- β 2 (expt 2) or MGF (expt 3). Cytosolic and membrane bound PK-C activity was determined as in [3]. PBL used for expts 1 and 2 are the same as in expts 1 and 2 in table 1. Values expressed as pmol 32 P/mg protein per min

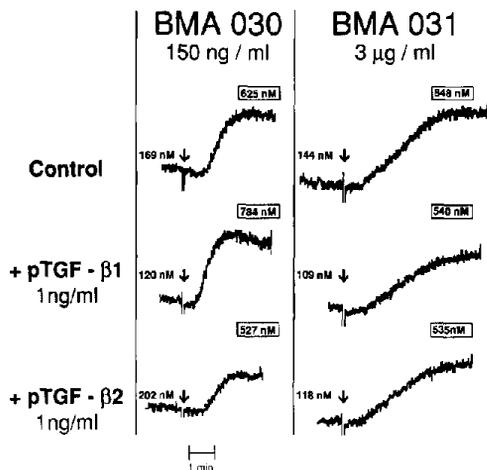


Fig.1. Human PBL were stimulated with the anti-CD3 mAb BMA 030 or the anti-TCR mAb BMA 031 in the absence or presence of 1 ng/ml pTGF- β 1 or - β 2. $[Ca^{2+}]_i$ was determined with fura-2. Boxed numbers, $[Ca^{2+}]_i$ after stimulation; unboxed numbers, basal $[Ca^{2+}]_i$. Arrow: addition of antibody.

at 37°C. The fura-2 fluorescence was monitored continuously using monochromator settings of 339 nm (excitation) and 492 nm (emission). The $[Ca^{2+}]_i$ levels were calculated using the general formula $[Ca^{2+}]_i = K_d (F - F_{min}) / (F_{max} - F)$; where K_d is the dissociation constant for Ca^{2+} binding to the indicator and F is arbitrary fluorescence units. For fura-2 a value of $K_d = 224$ nM was used [6]. F_{max} was determined by lysing cells with 0.01% Triton X-100. F_{min} was evaluated by adjusting the pH of the lysed cells to 10.5 with 60 nM Tris and addition of 7.5 mM EGTA.

3. RESULTS

$[^3H]$ Thymidine incorporation of human PBL induced by the anti-CD3 mAb BMA 030 was decreased by pTGF- β 1, - β 2 and MGF (table 1). The inhibition of $[^3H]$ thymidine incorporation was not complete and the maximum decrease was obtained at 1 ng/ml. The dose-response curves were similar. We have obtained equivalent results using the anti-CD3 mAb Lau-T3 (Stoeck, M. et al., submitted).

The effects of pTGF- β 1, - β 2 and MGF on the translocation of PK-C mediated by mAb 030 are shown in table 2. Whereas BMA 030 increased the membrane-bound fraction and decreased the cytosolic fraction of PKC, pTGF- β 1, - β 2 and MGF at 1 ng/ml neither influenced the translocation mediated by BMA 030 nor translocated PKC by themselves. Fig.1 shows the effect of pTGF- β 1 and - β 2 on the increase of $[Ca^{2+}]_i$ induced by mAb

BMA 030. Neither pTGF- β 1 nor - β 2 inhibited the increase of $[Ca^{2+}]_i$ induced by either antibody. Similar results were obtained using the mAb BMA 031 which recognizes the human TCR.

4. DISCUSSION

Our data show that pTGF- β 1, pTGF- β 2 and MGF decrease $[^3H]$ thymidine incorporation into anti-CD3-stimulated human lymphocytes without interfering with the increase of intracellular Ca^{2+} and the translocation of PK-C. This suggests that the transforming growth factors β exert their inhibitory function somewhere 'downstream' of the first events of cell activation. This concept is also supported by the data of Siepl et al. [7] and from our laboratory (Stoeck, M. et al., submitted) which show that TGF- β interferes with lymphocyte activation induced by a Ca^{2+} ionophore and a phorbol ester, a combination which circumvents the respective early events in lymphocyte activation [8]. In addition, IL-2-driven proliferation, which has been shown to be independent of PK-C translocation [9], is inhibited by TGF- β [4]. Furthermore, Chambard and Pouyssegur [10] have demonstrated that TGF- β inhibits the growth of fibroblasts without interfering with a series of 'early events'. In conclusion, TGF- β appears not to inhibit cell proliferation by interference with an event at the beginning of the activation cascade.

Besides their inhibitory and antiproliferative effects the transforming growth factors β also have stimulatory properties in several systems [4]. Here, it is of interest that Muldoon et al. [11,12] have reported that in Rat-1 cells TGF- β increases the IP_3 and IP_4 levels and enhances the EGF-mediated increase in $[Ca^{2+}]_i$. Furthermore Markovac and Goldstein [13] have shown that TGF- β activates protein PK-C in microvessels isolated from immature rat brain and suggested a role for TGF- β in brain microvascular differentiation. Thus, the effects of TGF- β on the early events could be dependent on the cell type and on its differentiation state.

Acknowledgements: The authors are indebted to Dr Robert Burk for continuous support. M.S. was in part supported by a grant from the Deutsche Forschungsgemeinschaft (DFG).

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