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Transforming growth factors β1 and β2 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

Michael Stoeck+, Henning Sommermeyer, Sylvia Miescher, David Cox*, Sefik Alkan* and Marta Szamel

+*Ludwig Institute for Cancer Research, Lausanne Branch, CH-1066 Epalinges, Switzerland, Dept of Molecular Pharmacology, Hannover Medical School, D-3000 Hannover, FRG* and **Pharmaceuticals Research Division, Ciba-Geigy Ltd, CH-4002 Basel, Switzerland*

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Porcine transforming growth factor 1 and 2 (pTGF-β1 and -β2) 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-β1, -β2 and milk growth factor, a bovine TGF-β2-like molecule, interfered with the increase of $[Ca^{2+}]_i$ and the activation of PK-C in human anti-CD3-stimulated lymphocytes.

Correspondence address: M. Stoeck, Laboratoire d'Oncologie, CHUV-BH 19-602, CH-1011 Lausanne, Switzerland

2. MATERIALS AND METHODS

2.1. *Chemicals*

Medium HB 102 (DuPont, Dreieich) was supplemented with 2 mM glutamine, 100 U/100 μg/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 β1 and β2 (pTGF-β1- and -β2) 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-β2. (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. *[^{3}H]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-β1, -β2 or MGF (10–0.001 ng/ml) was

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Table 1

Effect of pTGF-β1, pTGF-β2 and MGF on anti-CD3-induced [^{3}H]thymidine incorporation in human lymphocytes

		Experiment		
		1	2	3
∅		450[a] ± 73	241 ± 141	234 ± 32
+ BMA 030		14140 ± 825	53778 ± 869	36589 ± 2812
		pTGF-β1	pTGF-β2	MGF
	+ 5 ng/ml	9578 ± 945	31255 ± 2453	23905 ± 1843[b]
	+ 1 ng/ml	7204 ± 445	37735 ± 2990	23702 ± 1049
	+ 0.1 ng/ml	11093 ± 445	48746 ± 835	34163 ± 1555
	+ 0.01 ng/ml	13914 ± 899	48589 ± 2697	37804 ± 2039
	+ 0.001 ng/ml	14287 ± 1049	53195 ± 3009	40181 ± 1132

[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-β1, -β2 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 μCi/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-β1 or -β2 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-β1 or -β2. $[Ca^{2+}]_i$ 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 μM fura-2/AM. The labelled cells were washed in PBS and resuspended in PBS supplemented with 1 mM $CaCl_2$, 1 mM $MgCl_2$ 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-β1, TGF-β2 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
∅	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-β1 (expt 1), in pTGF-β2 (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

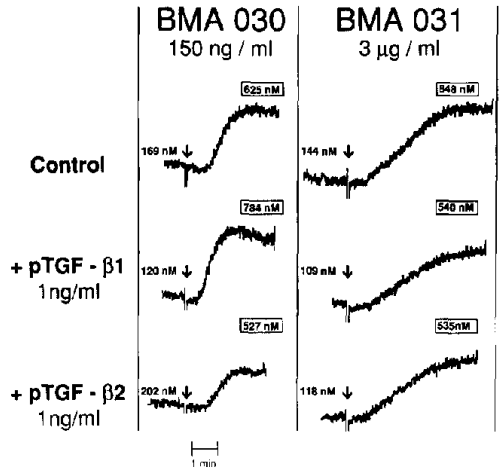

Fig.1. Human PBL were stimulated with the anti-CD3 mAb BMA 030 or the anti-TCR mAb BMA 031 in the absence or presence of 1 ng/ml pTGF-β1 or -β2. $[Ca^{2+}]_i$ was determined with fura-2. Boxed numbers, $[Ca^{2+}]_i$ after stimulation; unboxed numbers, basal $[Ca^{2+}]_i$. Arrow: addition of antibody.

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

[^{3}H]Thymidine incorporation of human PBL induced by the anti-CD3 mAb BMA 030 was decreased by pTGF-β1, -β2 and MGF (table 1). The inhibition of [^{3}H]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-β1, -β2 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-β1, -β2 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-β1 and -β2 on the increase of $[Ca^{2+}]_i$ induced by mAb BMA 030. Neither pTGF-β1 nor -β2 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-β1, pTGF-β2 and MGF decrease [^{3}H]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 Pouysségur [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.

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