Volume 227, number 2, 175–178 FEB 05506 January 1988

Rapid phosphorylation of a 27 kDa protein induced by tumor necrosis factor

Anne Hepburn, Dominique Demolle, Jean-Marie Boeynaems, Walter Fiers* and Jacques E. Dumont

IRIBHN, Campus Erasme, Route de Lennick, 808, 1070 Brussels and **Laboratory of Molecular Biology, State University of Ghent, Ghent, Belgium*

Received 23 November 1987

Tumor necrosis factor (TNF) has been shown to induce the phosphorylation of a 27 kDa protein in a time- and concentration-dependent manner in HeLa D98/AH_2, ME 180 and bovine aortic endothelial cells. This phosphorylation could be reproduced by the calcium ionophore, A23187. However, this phosphorylation was not observed in L929 cells, for which TNF is highly cytotoxic, suggesting that it might play a role in actions of TNF other than the induction of cell death.

Tumor necrosis factor; Phosphorylation

1. INTRODUCTION

Tumor necrosis factor (TNF), a protein secreted by monocytes/macrophages, causes the necrosis of solid tumors in vivo [1,2] as well as inhibiting the growth of several normal and tumoral cell lines in vitro [3,4]. TNF also has important immunomodulatory effects [5–7] and plays a role in endothelial cell metabolism, increasing the synthesis of prostacyclin [8] and modifying the hemostatic properties of these cells [9,10]. The sequence of events responsible for these effects of TNF is, as yet, unknown.

The regulation of many cell functions occurs via the phosphorylation of key proteins by specific protein kinases. The activities of these kinases are modulated by increases in cyclic nucleotide or cytoplasmic calcium levels and by the hydrolysis of phosphoinositides generating diacylglycerol (review [11]). It therefore appeared to be important to define the protein substrates phosphorylated upon addition of TNF to sensitive cells. The reproduction of a similar pattern of phosphorylation by other protein kinase activators might help to elucidate the intracellular signal induced by TNF. In this report, we show that, in some cells, the addition of TNF causes the rapid phosphorylation of a 27 kDa protein and that this effect is reproduced by the calcium ionophore, A23187. However, in the cell line L929, which is particularly sensitive to the cytotoxic action of TNF, this phosphorylation is not observed.

Correspondence address: A. Hepburn, IRIBHN, Bâtiment C, Campus Erasme, Route de Lennick, 808, 1070 Brussels, Belgium

2. MATERIALS AND METHODS

2.1. *Cells*

Bovine aortic endothelial cells were isolated from fresh bovine aortas obtained from the slaughterhouse. The endothelial cells were dissociated from the muscle layer by collagenase treatment as described previously [12,13]. These cells were cultured in a medium consisting of 3:1 DMEM/Ham's F12, 100 U/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B, supplemented with 20% fetal calf serum. They were used up to the fifth passage.

HeLa D98/AH_2 and ME-180 were originally obtained from cervical carcinomas. L929 cells are derived from a mouse fibrosarcoma. HeLa and L929 cells were grown in DMEM, ME-180 in MEM. Both media were supplemented with 10% fetal calf serum and penicillin and streptomycin as above.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

2.2. *Measurement of TNF-induced cytotoxicity*

Cells were seeded in 3.5 cm Petri dishes. In order to ascertain if TNF was cytotoxic or cytostatic, cells were incubated for 24 h with medium containing 1–5 μCi/ml [^{3}H]thymidine (Amersham). Tritium release was measured on aliquots of medium after incubation with varying concentrations of TNF for a further 24 h. The DNA content of dishes was measured as already described [14]. Prostacyclin release by bovine aortic endothelial cells was determined by the radioimmunoassay of its degradation product, prostaglandin 6-keto-$F_{1\alpha}$ [13].

2.3. *Phosphorylation of cell proteins*

Subconfluent cell layers were rinsed twice with Hank's buffer without phosphate then incubated for either 1 or 4 h in MEM without phosphate and 50 μCi/ml $^{32}PO_4$ (Amersham). The total medium concentration of phosphate was adjusted to 10^{-5} M with KH_2PO_4. Agents were added at specified times before the end of incubation. Cells were stopped by rinsing rapidly once with Hank's buffer without phosphate then adding 400 μl of a lysing buffer (62 mM Tris-HCl, pH 6.8, 10% glycerol, w/v, 5% β-mercaptoethanol, v/v, 2.3% SDS, w/v) containing protease inhibitors (0.1 mg/ml trypsin inhibitor, 2.5 μg/ml leupeptin, 50 μg/ml phenylmethylsulfonyl fluoride, w/v). SDS-PAGE was carried out on gradients of 6.5–20% polyacrylamide [15]. Molecular mass standards (Amersham) were used to calibrate each gel.

Recombinant human TNF was prepared as described [16].

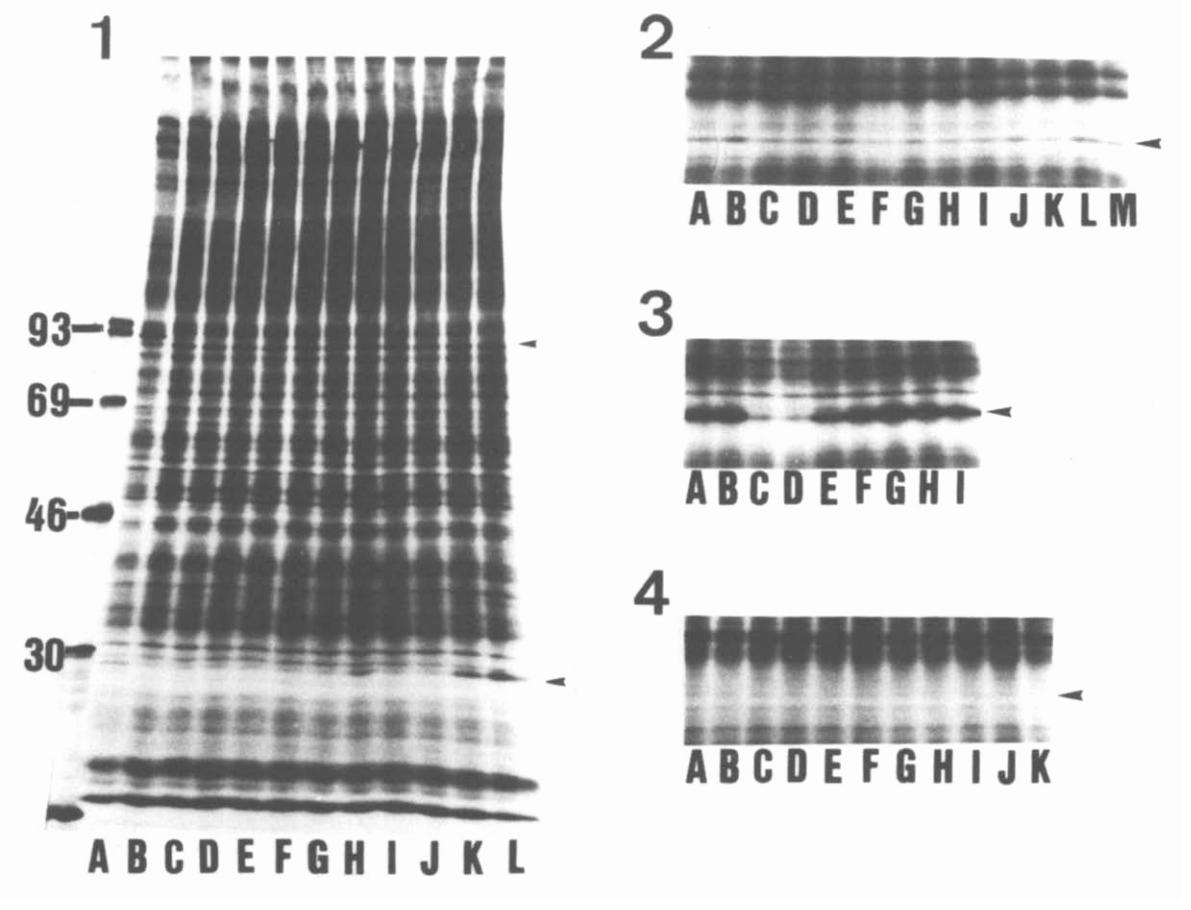

Fig.1. (Gel 1) HeLa D98/AH$_2$ cells (lanes): 5 μM A23187, 30 min (A); 0.2 U/ml TNF, 5 min (B); 0.2 U/ml TNF, 10 min (C); 0.2 U/ml TNF, 30 min (E); 20 U/ml TNF, 5 min (F); 20 U/ml TNF, 10 min (G); 20 U/ml TNF, 30 min (H); 2000 U/ml TNF, 5 min (J); 2000 U/ml TNF, 10 min (K); 2000 U/ml TNF, 30 min (L); control cells (D and I). (Gel 2) Bovine aortic endothelial cells (lanes): 1 μM A23187, 5 min (A); 1 μM A23187, 10 min (B); 0.2 U/ml TNF, 5 min (C); 0.2 U/ml TNF, 10 min (D); 0.2 U/ml TNF, 30 min (F); 20 U/ml TNF, 5 min (G); 20 U/ml TNF, 10 min (H); 20 U/ml TNF, 30 min (J); 2000 U/ml TNF, 5 min (K); 2000 U/ml TNF, 10 min (L); 2000 U/ml TNF, 30 min (M); control cells (E and I). (Gel 3) ME-180 cells (lanes): 0.2 U/ml TNF, 30 min (A); 20 U/ml TNF, 30 min (B); 2000 U/ml TNF, 5 min (E); 2000 U/ml TNF, 10 min (F); 2000 U/ml TNF, 30 min (G); 2000 U/ml TNF, 60 min (H); 2000 U/ml TNF, 4 h (I); control cells (C and D). (Gel 4) L929 cells (lanes): 5 μM A23187, 10 min (A); 5 μM A23187, 30 min (B); 0.2 U/ml TNF, 30 min (C); 20 U/ml TNF, 30 min (E); 2000 U/ml TNF, 5 min (F); 2000 U/ml TNF, 10 min (G); 2000 U/ml TNF, 30 min (I); 2000 U/ml TNF, 60 min (J); 2000 U/ml TNF, 4 h (K); control cells (D and H).

3. RESULTS AND DISCUSSION

The early events after TNF binding to its receptor are still unclear. As the increase in the levels of many intracellular second messengers will ultimately modify the phosphorylation of a usually discrete number of protein substrates, we decided to study the effects of TNF upon protein phosphorylation in several cells which are sensitive to the cytostatic/cytotoxic action of TNF. Fig.1 shows the increase in the phosphorylation of a 27 kDa protein which is induced by TNF in bovine aortic endothelial cells, HeLa D98/AH_2 and ME-180 cells: the cytotoxic effect of TNF on these cells is shown in table 1. The induction of this phosphorylation was rapid, usually occurring within 5 min of TNF addition, thereby making it one of the earliest effects of TNF which has been reported in the literature. Longer incubations (up to 4 h) using HeLa D98/AH_2 and ME-180 cells showed that the phosphorylation of this protein was maximal at approx. 30 min. Phosphorylation was also dependent upon the concentration of TNF used: in ME-180 cells, TNF increased the phosphorylation of the 27 kDa protein at a concentration (0.2 U/ml) which was insufficient to induce cell death (cf. table 1 and fig.1, gel 3, lane A).

Fig.1, gel 4, shows a representative experiment using L929 cells. In 5 separate incubations in duplicate, using TNF concentrations up to 2000 U/ml (causing approx. 80–90% cell death in 24 h) and for times ranging from 5 min to 4 h, no phosphorylation of a 27 kDa protein could be seen.

The phosphorylation of an apparently identical protein was reproduced by the calcium ionophore A23187 (1–5 μM, fig.1), except in L929 cells. This would suggest that this modification of the 27 kDa band might be due to calcium-dependent kinases. However, A23187 at concentrations as low as 30–100 nM in both endothelial (Demolle, unpublished) and HeLa D98/AH_2 cells (fig.1), induced the rapid phosphorylation of another protein of 92 kDa; this was not observed in TNF-treated cells. This suggests that TNF might produce a localized increase in cytosolic Ca^{2+}. The accessibility of the 27 kDa protein to TNF-induced phosphorylation might reflect its cellular location, e.g. near the TNF receptor. On the other hand, the fact that TNF does not induce the phosphorylation of the 92 kDa band could also imply that it does not cause an increase in cytosolic calcium and that

Table 1

Cells	TNF (U/ml)	DNA content	[^{3}H]Thymidine release	Prostacyclin release
Aortic endothelial	0	100 ± 3	100 ± 4	100 ± 11
	0.2	95 ± 4	91 ± 4	102 ± 18
	20	89 ± 3[a]	131 ± 1[c]	167 ± 56
	2000	84 ± 4[b]	194 ± 44[a]	253 ± 52[b]
HeLa D98/AH_2	0	100 ± 1	100 ± 19	
	0.2	102 ± 4	128 ± 21	n.d.
	20	91 ± 5	179 ± 30[a]	
	2000	78 ± 3[c]	263 ± 8[c]	
L929	0	100 ± 2	100 ± 18	
	0.2	98 ± 6	112 ± 10	n.d.
	20	66 ± 2[c]	258 ± 54[b]	
	2000	8 ± 1[c]	833 ± 45[c]	
ME-180	0	100 ± 5	100 ± 6	
	0.2	105 ± 1	98 ± 6	n.d.
	20	79 ± 4[b]	125 ± 12[a]	
	2000	56 ± 2[c]	198 ± 16[b]	

These values are from representative experiments and represent the percentages ± SD of triplicate dishes with respect to control cells. n.d., not determined. All statistics were performed using Student's t-test: [a] $p \leq 0.05$; [b] $p \leq 0.01$; [c] $p \leq 0.001$

the phosphorylation of the 27 kDa protein can be regulated by at least two different intracellular signals.

The phosphorylation of a 27 kDa protein in endothelial cells has been shown to be stimulated by high-density lipoproteins (HDL) [17], which stimulate the growth of these cells [18]. In addition, the phosphorylation of a 27 kDa protein can also be induced in Chinese hamster lung fibroblasts by various mitogens [19] and in platelets by thrombin [20]. Our results show that TNF, which is cytotoxic for aortic endothelial cells in these conditions ([21] and table 1), also induces the phosphorylation of an apparently similar 27 kDa protein, with an identical time course as the mitogenic agents. As both HDLs and TNF have other effects on endothelial cells, e.g. stimulation of prostacyclin formation ([22] for HDLs, [8] and table 1 for TNF), this protein is perhaps implicated in the regulation of these processes rather than in cell multiplication. Future studies should determine if the 27 kDa proteins phosphorylated in response to mitogenic agents or TNF are in fact identical and if the phosphorylation involves the same amino acid.

The fact that the 27 kDa band is not phosphorylated in L929 cells, which are highly sensitive to the cytotoxic action of TNF, suggests that this phosphorylation is not necessary for cell death.

Phospholipase A_2 might play a role in the cytotoxicity induced by TNF in L929 cells [13,23,24]. Recently, Clark et al. (cited in [25]) have found that TNF can induce the synthesis of a 28 kDa protein in bovine endothelial cells which is related to melittin and has phospholipase A_2-stimulatory effects. The relationship between this protein and the 27 kDa protein phosphorylated in our studies merits investigation. Experiments using TNF-sensitive and resistant clones of the same cell line should help to determine if this phosphorylation, like the induction of a 26 kDa protein by TNF [26], is involved in the resistance of cells towards the cytotoxic action of TNF.

Acknowledgements: The authors would like to thank B. Genth and M. Pfreundschuh for the kind gift of the ME-180 cells and J. Tavernier for the TNF used in this study. They also thank C. Erneux for many helpful discussions. This work was supported by grants from the 'Actions Concertées' and the 'Caisse Générale d'Epargne et de Retraite'.

REFERENCES

[1] Carswell, E.A., Old, L.J., Kassel, R.L., Green, S., Fiore, N. and Williamson, B. (1975) Proc. Natl. Acad. Sci. USA 72, 3666–3670.
[2] Creasey, A.A., Reynolds, M.T. and Laird, W. (1986) Cancer Res. 46, 5687–5690.
[3] Sugarman, B.J., Aggarwal, B.B., Hass, P.E., Figari, I.S., Palladino, M.A. and Shepard, H.M. (1985) Science 230, 943–945.
[4] Ruggiero, V., Latham, K. and Baglioni, C. (1987) J. Immunol. 138, 2711–2717.
[5] Talmadge, J.E., Tribble, H.R., Pennington, R.W., Phillips, H. and Wiltrout, R.H. (1987) Cancer Res. 47, 2563–2570.
[6] Ming, W.J., Bersani, L. and Mantovani, A. (1987) J. Immunol. 138, 1469–1474.
[7] Scheurich, P., Thoma, B., Ücer, U. and Pfizenmaier, K. (1987) J. Immunol. 138, 1786–1790.
[8] Kawakami, M., Isibashi, S., Ogawa, H., Murase, T., Takaku, F. and Shibata, S. (1986) Biochem. Biophys. Res. Commun. 141, 482–487.
[9] Nawroth, P.P. and Stern, D.M. (1986) J. Exp. Med. 163, 740–745.
[10] Bevilaqua, M.P., Pober, J.S., Majeau, G.R., Fiers, W., Cotran, R.S. and Gimbrone, M.A. (1986) Proc. Natl. Acad. Sci. USA 83, 4533–4537.
[11] Hunter, T. (1987) Cell 50, 823–829.
[12] Boosyse, F.M., Sedlak, B.J. and Rafelsen, M.E. (1975) Thromb. Diathes. Haemorrh. 34, 825–839.
[13] Van Coevorden, A. and Boeynaems, J.M. (1984) Prostaglandins 27, 615–626.
[14] Hepburn, A., Boeynaems, J.M., Fiers, W. and Dumont, J.E. (1987) Biochem. Biophys. Res. Commun., in press.
[15] Laemmli , U.K. (1970) Nature 261, 558–560.
[16] Tavernier, J., Fransen, L., Marmanout, A., Van Der Heyden, J., Müller, R., Ruysschaert, M.R., Van Vliet, A., Bauwden, R. and Fiers, W. (1987) in: Lymphokines (Pick, E. ed.) vol.13, pp.181–198, Academic Press, New York.
[17] Darbon, J.M., Tournier, J.F., Tauber, J.P. and Bayard, F. (1986) J. Biol. Chem. 261, 8002–8008.
[18] Tauber, J.P., Cheng, J. and Gospodarowicz, D. (1980) J. Clin. Invest.
[19] Chambard, J.C., Franchi, A., Le Cam, A. and Pouysségur, J. (1983) J. Biol. Chem. 258, 1706–1713.
[20] Chambard, J.C. and Pouysségur, J. (1983) Biochem. Biophys. Res. Commun. 111, 1034–1044.
[21] Frater-Schröder, M., Risau, W., Hallman, R., Gautschi, P. and Böhlen, P. (1987) Proc. Natl. Acad. Sci. USA 84, 5277–5281.
[22] Fleisher, L.N., Tall, A.R., Witte, L.D., Miller, R.W. and Cannon, P.J. (1982) J. Biol. Chem. 257, 6653–6655.
[23] Matthews, N., Neale, M.L., Jackson, S.K. and Stark, J.M. (1987) Immunology 62, 153–155.
[24] Suffys, P., Beyaert, R., Van Roy, F. and Fiers, W. (1987) Biochem. Biophys. Res. Commun., in press.
[25] Clark, M.A., Conway, T.M., Shorr, R.G.L. and Crooke, S.T. (1987) J. Biol. Chem. 262, 4402–4406.
[26] Defilippi, P., Poupart, P., Tavernier, J., Fiers, W. and Content, J. (1987) Proc. Natl. Acad. Sci. USA 84, 4557–4561.