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A protein produced by a monocytic human cell line can induce apoptosis on tumor cells

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Abstract

A serum-free medium conditioned by U937, a human cell line of monocyte/macrophage origin, was found capable of inducing apoptosis on exponentially growing U937 cells themselves (autocrine suicide). The apoptosis-inducing agent is a macromolecule and possibly a protein (SKT factor), with a relative molecular mass in the range of 18–25 kDa. All human tumor cell lines examined have been induced to apoptosis with high efficiency, whereas non transformed human lymphocytes and monocytes are insensitive to the apoptosis-inducing activity; moreover, partially differentiated U937 are not killed but induced to full maturation. These observations suggest that the SKT factor could possibly be a cytokine with a specific cytotoxic tropism, that resembles in many respects the cytokine tumor necrosis factor (TNF), even though no TNF is detectable in the conditioned medium.

Key words: Monocyte/macrophage; Cytokine; Apoptosis; Tumor cell; Differentiation

1. Introduction

Cell killing by protein factors has long been described, and concerns unwanted cells within the organism, such as transformed or exogenous cells. Cell killing can be achieved by damaging the target cells, as in the case of perforin, a protein that aspecifically produces channels in the plasma membrane, leading to necrosis [1]. However, a more precisely 'targeted' mode of cell killing can be achieved by the acting proteins throughout the stimulation of specific receptors on the target cells, that are thus induced to commit suicide [2]. In these instances cell death is the result of a cross-talk between cells, and the mode of cell death is almost invariably apoptosis [3]. Indeed apoptotic cell death avoids the inflammation process that always accompanies necrosis [4], and is characteristic of 'physiological', programmed, cell death [5–8]. The proteins involved in this type of cell killing are functionally cytokines [9]; the better studied example is the tumor necrosis factor (TNF) [10–13], which induces apoptosis preferentially on tumor cells which possess the specific receptors (TNFr) [14–16], through mechanisms that have not been completely elucidated so far [17]. TNFrs belong to a family of integral membrane proteins that are able to transduce a signal of suicide once specifically activated, that include also the Apo-1 and the Fas antigen [18–21] (indeed, they have been shown to be identical [22]). Also the receptor for nerve growth factor belongs to the same family, even though it has an antithetic role, since it protects cells from apoptosis [23]. Interestingly, TNF itself can induce cell proliferation (as opposed to cell killing) on non-transformed fibroblasts [24] and other cell types [25], and can also act as a differentiating agent [26–28], depending on the target cells. Indeed, the messages brought about by these cytokines, viewed in the context of the whole organism, are apparantly ambiguous, since the same message can induce proliferation, differentiation or apoptosis. We describe here the first observations about a novel factor, possibly a protein, that is indeed capable of exerting either a differentiative or an apoptosis-inducing action depending on the target cells.

2. Materials and methods

2.1. Cell culture and treatments

U937 cells were kindly provided by Dr. E. D'Ambrosio, Dip. Medicina Sperimentale, C.N.R., Roma. All the cells were cultured in RPMI medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin and streptomycin and 10% FCS (unless otherwise indicated), and kept in a controlled atmosphere (5% CO_2) incubator at 37°C.

Apoptotic activity inhibition treatment: cells in conditioned medium have been treated with the following chemicals: Cycloheximide, 10 μg/ml; 3-aminobenzamide, 5 mM; PMSF, 1 mM.

Differentiation inducing treatment: cells were incubated for 48 h with the following compounds: 1.2 mM Dimethylsulfoxide (DMSO); 0.5 μM 12-*O*-tetradecanoylphorbol 13-acetate (TPA); 10 mM butyric acid; or have been kept in alkaline medium (pH 8–8.5) for 48 h. When appropriated (see section 3), the partially differentiated stage (pseudo-monocyte stage) has been controlled by the ability to reduce NitroBlue-tetrazolium (NBT) [29], whereas in the fully differentiated stage (pseudo-macrophage) this characteristic is suddenly lost, and the emission of

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pseudopodes is observed and attachment to the substrate of the culture flask [30].

2.2. Analysis of apoptosis

Apoptosis was characterized by DNA fragmentation to give a ladder-like pattern, and nuclear fragmentation in several smaller fragments, ranging in number from 2 up to > 20 per cell, detectable by optical microscopy on slides of hematossilyn-stained cells.

Preparation and staining of slides: 2×10^5 cells, fixed in 4% paraformaldeide, were loaded on a gelatinized slide, stained with hematoxylin, and analyzed for direct optical microscopy.

Analysis of DNA: 10^6 cells were lysed in a buffer containing 10 mM EDTA, 100 mM Tris pH 8, 0.5% sodium lauroyl sarkosine, 200 μg/ml proteinase K. Nucleic acids were extracted by phenol-(chloroform/isoamylic alcohol 24:1), ethanol precipitated, incubated in 100 μg/ml RNase A 60 min at 37°C. The purified DNA is loaded on a 1.5% agarose gel in TAE buffer, stained with 10 μg/ml ethydium bromide, and visualyzed on a 254 nM UV transilluminator.

Quantification of apoptosis: the fraction of cells with a fragmented nucleus among the total cell population, was calculated on the hematoxylin-stained slides, counting at least 100 cells in at least 3 random selected fields (a comparison with cytofluorimetric analysis is to be described elsewhere).

2.3. Conditioned medium fractionation and activity assay

To foetal calf serum-containing conditioned medium was added $(NH_4)_2SO_4$. The resulting precipitates at 20, 40, 60 and 80% $(NH_4)_2SO_4$ were collected by centrifugation, dissolved in a small volume of phosphate-buffered saline (PBS) and dialyzed against serum-free medium. The activity assay has been performed by calculating the fraction of apoptotic cells (see above) after 4 and 18 h of incubation of exponentially growing cells in the dialyzed fractions.

Serum-free conditioned medium was gel-filtered on a Sephadex G-100 column (1.6 × 30 cm) with 0.3 M NH_4HCO_3. In order to test the SKT factor activity, chromatographic fractions were collected, lyophilized, dissolved in serum free medium, and pools of four contiguous fraction were applied on exponentially growing U937. The activity assay has been performed by calculating the fraction of apoptotic cells (see above) after 4 and 18 h of incubation.

2.4. TNF detection

U937 conditioned medium was assayed for TNFa with an ELISA assay, by a commercially available kit with a detection limit of 25 pg/ml.

3. Results

3.1. Induction to apoptosis by conditioned medium

Under several circumstances (which we are still analyzing) U937 cells respond by undergoing an as yet 'unexplainable' apoptosis (Fig. 1): in these instances they condition the culture medium which, independently of the presence of FCS, can induce exponentially growing U937 to apoptosis. Fig. 2 shows the characteristics of apoptotic (vs. normal) U937, that is: nuclear fragmentation (Fig. 2a), and DNA fragmentation in a ladder-like pattern (Fig. 2b). Fig. 3 describes the kinetics of the induction to apoptosis by U937-conditioned medium, obtained by calculating the fraction of cells with apoptotic morphology (see Fig. 2a) among the total cell population (see section 2). The presence of two separate peaks, observed in all the experiments performed, has been confirmed by cytofluorimetric analysis (to be described elsewhere), and is characteristic of the apoptosis induced by this conditioned medium. Indeed, in the case described here, U937 that undergo apoptosis during the

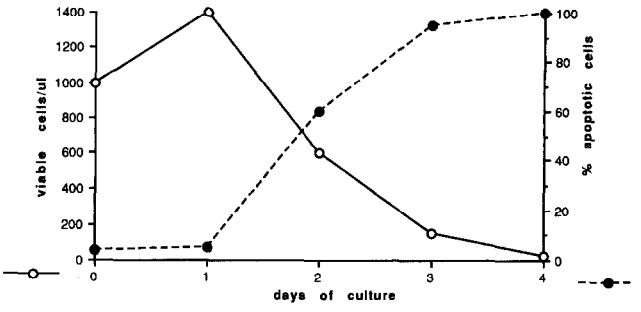

Fig. 1. Serum-free growth curve of U937, following treatment as to produce apoptosis-inducing activity.

first wave are rapidly removed from the culture, whereas in the case of apoptosis induced by other agents (i.e. stress conditions) the apoptotic cells do not disappear from the culture, and can thus accumulate in the medium [31].

The two peaks should be the result of the same inducing agent, since they have been obtained even with partially fractionated conditioned medium (see below); however, we cannot exclude that more than one apoptotic inducing activity with different effects is present in the conditioned medium.

3.2. The apoptosis-inducing activity is a macromolecule and possibly a protein

We observed that the activity is maintained after dialysis against serum-free medium, showing that the agent is a macromolecule. Moreover, it is precipitable by ammonium sulphate (AS); indeed upon AS fractionation, only a slight activity was present in the fractions precipitated at concentrations below 80%, while 100% induction to apoptosis was obtained with the protein fractions precipitated with 80% AS. This suggests that the apoptosis inducing activity is a protein, that we have called the SKT factor, since other possible effector macromolecules (i.e. sugars or lipids) are not precipitable by AS [32].

We have performed a preliminary purification of the SKT factor activity from serum-free conditioned medium by gel permeation chromatography over a G-100 Sephadex column. The two experiments performed have shown that the apoptosis-inducing activity is eluted in a range of relative molecular mass of about 18–25 kDa, and is confined to a few adjacent fractions (among 80 collected) that give 100% induction to apoptosis, whereas all the other fractions give background values (Fig. 4)

3.3. Effect of conditioned medium on various cell types and differentiative stages

We observed that all of the four human tumor cell lines tested so far were induced to apoptosis by the conditioned medium containing the SKT factor (Table 1). Also freshly explanted human monocytes (HM), still in-

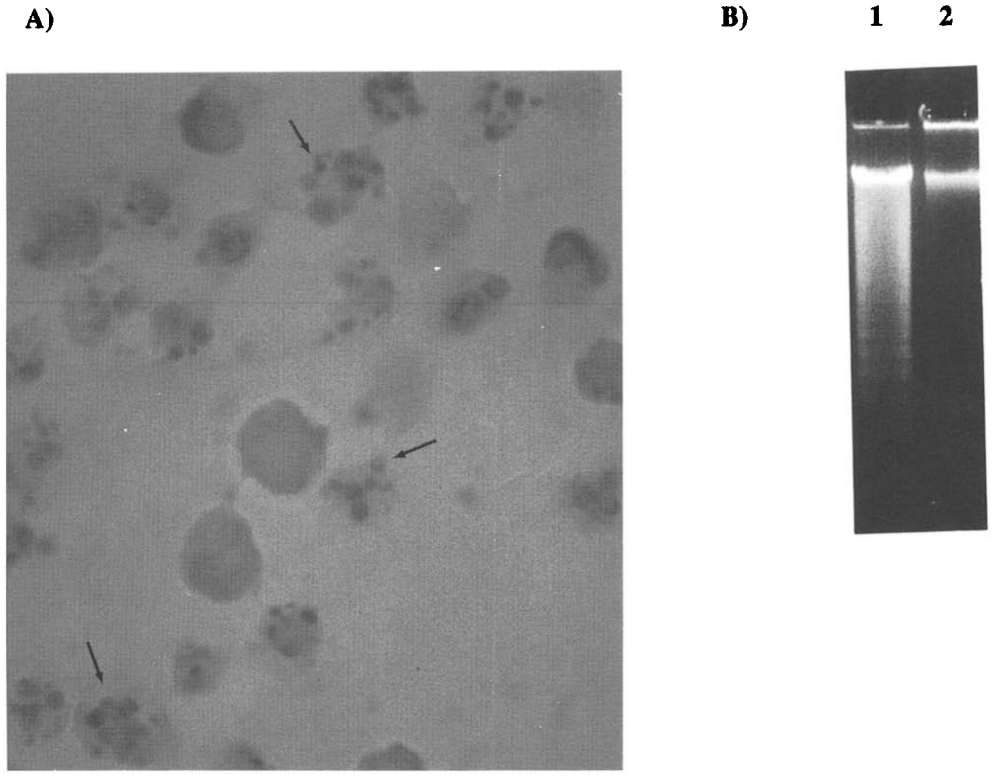

Fig. 2. Characteristics of apoptotic U937, as appear after 4 h incubation in conditioned medium. (A) Morphology of apoptotic cells (arrows), with highly fragmented nuclei; and normal, viable U937. (B) DNA analysis of apoptotic (1) and viable (2) U937, showing the characteristic DNA fragmentation of apoptotic cells.

differentiated, are induced to apoptosis. Instead, once differentiated by the cytokine macrophage-colony stimulating factor (M-CSF), HM lost sensitivity and survived the treatment (Table 1). Interestingly, also the sensitivity of U937 cells to the cytotoxic activity of SKT can be modulated by the differentiative state of the cells.

U937 were shown to be induced to differentiate by several treatments, to give slightly different differentiated states [30]. We have used three known differentiative agents: dimethylsulfoxide (DMSO); 12-*O*-tetradecanoylphorbol 13-acetate (TPA); butyric acid; and a novel differentiation-inducing treatment, that is the incubation of exponentially growing U937 in alkaline medium (pH 8–8.5) for 48 h (to be described elsewhere). TPA, butyric acid, and alkaline medium, all induce on U937 the ability to reduce NBT, a known marker of the monocytic differentiative stage (see section 2), though the induced cells did not look alike (i.e. TPA induces terminal differentiation with the emission of pseudopods; DMSO induce a slight different cell morphology and not the ability of reducing NBT), suggesting that the different treatments end up with different differentiated states.

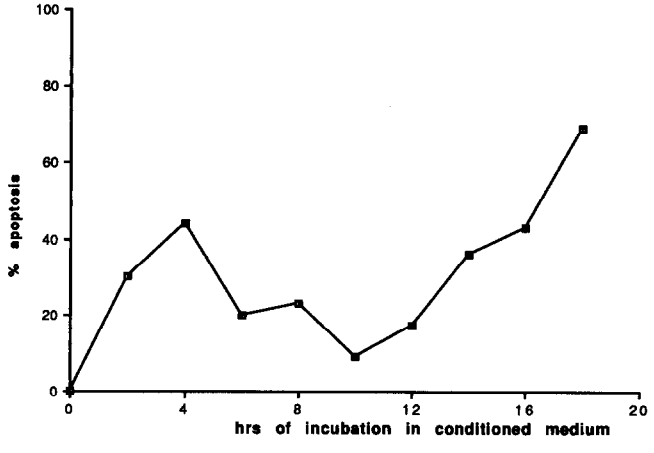

Fig. 3. Kinetics of the induction to apoptosis by U937-conditioned medium on exponentially growing U937 cells (one of several experiments is shown).

Table 2 shows that the apoptosis-inducing activity of SKT, on U937 induced to differentiate by different agents, is strongly reduced in three out of four treatments.

Moreover, upon differentiation induced by alkaline medium, SKT cytotoxic activity is converted into an activity capable of inducing full maturation: alkaline medium-treated rounded, floating, NBT positive U937, upon incubation with the SKT factor become attached to the substrate, show pseudopods, and have lost the capacity of reducing NBT, all characteristics of differentiated macrophages.

3.4. Functional analysis of the SKT factor

In order to gain an insight in the mechanism of action

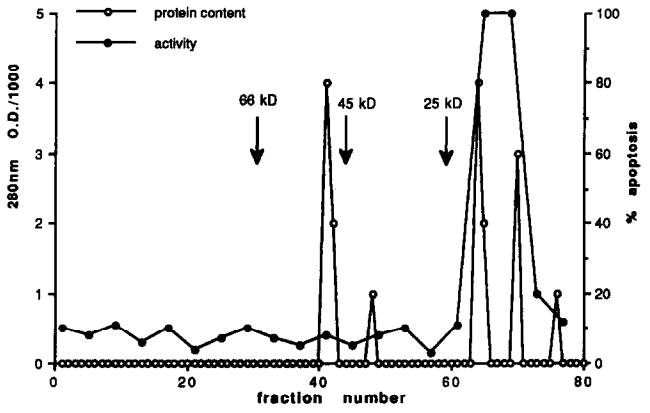

Fig. 4. Gel filtration of U937-conditioned medium. Protein profile and apoptosis-inducing activity after G-100 Sephadex column filtration (one of two independent experiments is shown).

of the SKT factor, we tried to block its apoptosis-inducing activity. For this purpose, we tested the action of compounds that are known to block or inhibit apoptosis induced by other means, by treating exponentially growing cells before and during incubation in the conditioned medium. The protein synthesis inhibitor cicloheximide (CHX), and the poly-ADP-ribosyl-transferase inhibitor, 3-aminobenzamide (3-ABA), both shown to inhibit some forms of apoptosis [31,33–36], did not have any effect on blocking the apoptosis-inducing activity of the SKT factor, nor did the Ca^{2+} ion-chelating agent EGTA. Instead, we could block it by using the protease inhibitor PMSF. This is in line with several findings describing cytotoxic proteins with site-specific protease activity [37,38]. However, this does not necessarily mean that the SKT factor is in itself a protease, but rather that a protease is likely to be necessary in the chain of events leading to the induction to apoptosis (indeed PMSF can enter inside the cells): it is known that the apoptosis-inducing activity of TNF is sensitive to the use of protease inhibitors, even though TNF does not have any protease activity [39].

The SKT factor and TNF indeed share also other characteristics: they are produced by the same cell types, have a similar molecular weight [40] and similar specific cytotoxicity, and can induce differentiation or apoptosis depending on the target cells [41]; however, they are not the same molecule, for the following reasons. First, no TNF was detectable in the conditioned medium by ELISA; second, the kinetics of induction to apoptosis by TNF is much slower than the SKT's (indeed we began to detect it at 18 h post-incubation, not shown); third, the Epstain-Barr virus positive (EBV^+) lymphoma Raji, insensitive to the cytotoxic effect of TNF (indeed they lack the TNF receptor) [42], are induced to apoptosis by the SKT factor (see Table 1); fourth, TNF is recovered as a 40–45 kDa trimer from gel filtration [10,43], whereas the profile of SKT elution shows that the cytotoxic activity is eluted as a smaller molecule (Fig. 4).

4. Discussion

The data presented here suggest that the SKT factor, capable of inducing apoptosis on tumor cells, is a protein, and is produced by the human cell line of monocytic origin U937. Indeed, it is not the result of an alteration of a seric protein, since it is present even in serum-free conditioned medium. Other cytotoxic factors, of monocyte/macrophage or lymphocyte origin, can induce apoptosis on target cells, such as leukoregulin [44] and a serine esterase [37], released by cytotoxic T lymphocytes, fragmentin, released by natural killer cells [45], an arginase released by macrophages [38], TNFα, TNFβ (also known as lymphotoxin) [11,46]. However, the SKT factor differs from any of these proteins either by mode of action or molecular weight (i.e. all the isoformes of the macrophages-differentiating CSF-1 cytokine are larger than SKT), so that we can exclude that they are identical to SKT. Moreover, we can also exclude identity to TNF (see section 3), even if its characteristics look very similar to those of SKT. Many cytokines, known for different properties, such as interferon γ [47], can act as cytotoxic effectors in some instances; we are analyzing whether the SKT factor could be an already known cytokine, although a behaviour such as that described for SKT has not been described so far; this leads us to believe this could be a novel factor. Alternatively, it could arise from the specific proteolysis of a cytokine that could be non-cytotoxic in the native state, to give a product of homogenous size. The Fas-antigen, which exerts an apoptosis-inducing function similar to TNFr's, responds to the stimulation of a ligand that has not yet been identified, though its excistance has been inferred by genetic analysis of the *gld* murine lymphoproliferative pathology [48].

Table 1
Sensitivity to the cytotoxic activity of SKT factor. Analysis on various cell types and cell lines

SKT sensitive cells	SKT non-sensitive cells
Ramos (human lymphoma, EBV^-)	Human quiescent blood lymphocytes
Raji (human lymphoma, EBV^+)	Human monocytes, differentiated with M-CSF
HeLa (human epithelial tumor cell line)	U937, differentiated
U937 (human monocytes tumor cell line), undifferentiated	
Human monocytes, undifferentiated	

Table 2
Quantification of apoptosis by the SKT factor on U937 induced to differentiation

	C	Alkaline medium	DMSO 1.2 mM	TPA 0.5 μM	Butyric acid 10 mM
Fresh medium	1	12	9	25	4
Conditioned medium (18 h)	63	8*	21	37	100

The values indicate the fraction of apoptotic cells at the end of each treatment (see section 2). All differentiating treatments lasted 48 h, then differentiated cells were incubated with either fresh or conditioned medium for a further 18 h.
*All viable cells look terminally differentiated (see section 2).

It would sound appealing that the ligand of the Fas antigen would be the SKT factor, since the mode, specificity, and kinetics of SKT cytotoxic activity are very similar to those induced by the anti-Fas antibody on Fas antigen-bearing cells.

SKT's selective behaviour, that is induction to apoptosis on indifferentiated cells, induction to full maturation on partially differentiated cells, recalls the specific cytotoxicity TNF exerts on tumor vs. normal cells. What could be the reason for this different cell response? In the case of TNF, the selectivity can be ascribed for some cells to the lack of specific receptors (i.e. EBV^+ lymphomas), but in the instances where it acts as a growth or differentiation factor, the receptors (the same receptors), are present on the surface of target cells, and are correctly internalized [49,50]. The current interpretation is that normal cells have a 'protector' protein(s) that tumor cells cannot make anymore: indeed, normal cells treated with cycloheximide become sensitive to the cytotoxic action of TNF, as though the protein synthesis inhibition had eliminated the 'protector' protein(s) [51]. In the SKT/U937 system the induction to differentiation, usually associated to the loss of the malignant phenotype, could be paralleled by the reappearance of a factor present in normal, differentiated monocytes/macrophages, that could turn the SKT cytotoxic signal into a physiological maturation signal.

All these considerations suggest that SKT action is biologically very specific, and could participate in the cross-talk between cells. It seems interesting to further analyze the factor, purifying it to homogeneity, and get the gene that encodes it, and specific antibodies for its localyzation within a healthy organism, as well as in organisms with defined pathologies. Moreover, it could be interesting to evaluate the SKT factor activity in cells of non-human origin, in order to obtain a system in which SKT functions could be analyzed in vivo. Indeed, it could have a relevance in tumor cell elimination or in pathologies involving macrophage terminal differentiation.

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