Fas antigen signals proliferation of normal human diploid fibroblast and its mechanism is different from tumor necrosis factor receptor

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Abstract Recent cloning of the cDNA for Fas/Apo-1 and its ligand has revealed that they belong to the tumor necrosis factor (TNF) receptor and TNF family, respectively, and play an important role in apoptosis (programmed cell death). Like TNF, antibodies against the Fas antigen (anti-Fas) have been shown to be cytotoxic to Fas-expressing cells. Whether Fas, like TNF receptor, also mediates proliferation of normal human diploid fibroblasts (HDF), is not known. In this study, we show that HDF expresses Fas antigen and the engagement of this antigen signals proliferation of these cells in a dose-dependent manner. Unlike TNF receptor, however, Fas-mediated proliferation of HDF could not be blocked by orthovanadate, a tyrosine phosphatase inhibitor. The difference in the signaling was further evident from our observation that TNF induced the expression of interleukin-6 but anti-Fas did not. Overall, our results demonstrate for the first time that besides cell killing, Fas also mediates proliferation of HDF and that its mechanism is different from that of TNF receptor.

Key words: Fas/Apo-1; TNF; Proliferation; IL-6; Fibroblast; Protein tyrosine phosphatase; Apoptosis

1. Introduction

Tumor necrosis factor (TNF) is a 26 kDa type II transmembrane non-glycoprotein of 233 amino acid residues whose 17 kDa extracellular domain (157 amino acids) is secreted by a number of different cell types, including activated macrophages. TNF is cytotoxic to some tumor cells but stimulates the proliferation of other cell types (for references see [1]). It is also involved in a wide variety of other biological actions [1,2]. TNF interacts with cells through two distinct receptors, p60 and p80, whose extracellular domains are highly homologous in amino acid sequence but whose cytoplasmic domains are quite distinct [3].

Another member has been added to the tumor necrosis factor (TNF) receptor superfamily with the recent cloning of the cDNA for human Fas/Apo-1 antigen (Fas), which revealed that it is a transmembrane 319 amino acid polypeptide with a molecular mass of 36 kDa [4]. The isolation of the ligand that interacts with human Fas has shown that it is a type II transmembrane polypeptide of 278 amino acids with molecular mass of 31 kDa and that it, too, is a member of the TNF family [5]. Its putative extracellular domain consists of 179 amino acids and contains four potential N-glycosylation sites [5]. The Fas antigen has been shown to be expressed by various human cells, including myeloid cells, T-lymphoblastoid cells, and diploid fibroblasts [6], whereas the Fas ligand is expressed by activated splenocytes, thymocytes, and certain non-lymphoid tissues such as testis [5].

By using human diploid fibroblast cells as an immunogen, Yonehara and colleagues developed a Fas mAb, anti-Fas, that is cytolytic to Fas-expressing cells [6]. Since the Fas antigen and TNF receptor were co-down-regulated, it was suggested that the Fas antigen may be associated with the TNF receptor. A similar antibody called Apo-1 has been described that also recognizes Fas [7]. Although the extracellular domain of the p60 form of TNF receptor displays as much amino acid sequence homology (24–30%) to the Fas antigen as to the p80 TNF receptor, their cytoplasmic domains are quite distinct. This has led to the hypothesis that p60, p80, and Fas must transduce distinct signals, even though all three receptors can mediate cell killing. A close examination of the cytoplasmic domain of the p60 receptor and Fas has led to the identification of a 45-amino acid region that displays 51% homology between the two [4]. Since the deletion of this domain in both the p60 receptor and Fas abolished their ability to mediate cell killing, this domain has been referred to as the 'death domain' [8,9].

Besides cell killing, TNF receptors have been shown to mediate proliferation of normal human diploid fibroblast (HDF) [2,10], but whether Fas can also signal this activity is not known. To help resolve that question we compared TNF with anti-Fas. Our results indicate that Fas antigen, like TNF receptor, mediates proliferation of HDF but unlike TNF, the proliferative signal induced by Anti-Fas is insensitive to orthovanadate. Also, unlike TNF, we found that treatment of HDF with anti-Fas does not lead to IL-6 production. Our results suggest that the death domain present in both p60 and the Fas receptors must mediate not only cytotoxic but also proliferative signals.

2. Materials and methods

2.1. Materials

RPMI-1640 was obtained from Whittaker MA Bioproducts (Walkersville, MD). Fetal bovine serum (FBS) and gentamicin were from Gibco (Grand Island, NY). Bacteria-derived recombinant human TNF (specific activity 5×10^7 U/mg) was kindly supplied by Genentech Inc. (South San Francisco, CA). Anti-Fas was a generous gift from Dr. Shin Yonehara, The University of Kyoto (Kyoto, Japan). Other chemicals and biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Human diploid fibroblasts (HDF) were kindly provided by Dr. Olivia Perreira-Smith (Baylor College of Medicine, Houston, TX). Cells were routinely grown in RPMI 1640 medium supplemented with glutamine (2 mM), gentamicin (50 μg/ml), and fetal bovine serum (10%).

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Abbreviations: TNF, tumor necrosis factor; p60 (also referred to as p55), TNF receptor I or TNF receptor type B; p80 (also referred to as p75), TNF receptor II or TNF receptor type A; IL-6, interleukin-6; Fas antigen is also referred as Apo-1 or CD95.

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2.2. Expression of Fas antigen, p60, and p80 receptor by flow cytometry

Expression of Fas antigen was determined by using Anti-Fas mAb as described by Yonehara et al. [6]. Briefly, 0.2×10^6 cells were incubated with 1 μg of anti-Fas in a final volume of 50 μl PBS containing 5% FBS and 0.01% sodium azide for 1 h on ice. After two washes with ice-cold PBS containing 5% FCS and 0.01% NaN_3, 10 μg/ml of fluorescein-conjugated affinity purified F(ab)2 fragment goat anti-mouse IgM (Capple, Organon Tekemika, West Chester, PA) were added in a final volume of 50 μl and incubated on ice for an additional 1 h. Cells were washed twice and resuspended in PBS containing 1% paraformaldehyde. Cells stained by second antibody alone were used to determine background fluorescence. Expression of p60 and p80 was determined by using htr-9 and utr-1 [8] respectively, at a concentration of 20 μg/ml followed by staining with second antibody (FITC-conjugated goat F(ab)'2 anti-mouse IgG (H + L) (Caltag, San Francisco, CA). FITC fluorescence was detected on a log scale using a FACScan analyzer (Becton-Dickinson, Mountain View, CA).

2.3. Fibroblast proliferation assays

Cell growth-stimulatory assays were carried out essentially according to the procedure described by Vilcek et al. [10]. Briefly, confluent human diploid foreskin fibroblasts (at passage level 12–15) were used for cell growth-stimulatory assays. To determine the effect of TNF and anti-Fas, cells (8×10^3/well) were plated in 0.1 ml of the medium (RPMI-1640 + 10% FBS) in 96-well Falcon plates. After overnight incubation in a CO_2 incubator at 37°C, the medium was removed and a serial dilution of the TNF or anti-Fas was layered in 0.1 ml of RPMI-1640 medium. During the last 24 h of a 72 h incubation, tritiated thymidine (6.7 Ci/mmol) was added to each well (0.5 μCi/well). The culture medium was then removed, the wells were washed twice with PBS, the cells suspension was harvested with the aid of a Packard Micromate 196 cell harvester onto a glass fiber filter, and radioactivity bound to the filter was measured in a Packard Matrix 9600 direct beta counter (Packard Co., Meriden, CT). Relative cell viability was calculated as the amount of thymidine incorporated in treated cells divided by that in the untreated cells and expressed as a percentage. The cell proliferation obtained with thymidine incorporation was found to correlate with cell number monitored by Trypan blue exclusion as well as by the Crystal violet dye uptake method. Although for most experiments confluent cell cultures (8×10^3 cells/well) were used, subconfluent cultures (4×10^3 cells/well) also gave similar results.

2.4. Determination of interleukin-6

HDF at 0.1×10^6 cells/ml were incubated in 24-well plates with TNF (20 ng/ml) or anti-Fas (20 ng/ml) for 24 h, and then the supernatants were collected. The presence of IL-6 was determined by using the IL-6-dependent murine B cell hybridoma B-9 as described previously [11]. Briefly, 2×10^3 cells were incubated in 0.2 ml of RPMI-1640 containing 10% FBS in 96-well plates with different concentrations of either IL-6 or IL-6-containing supernatants for 96 h, and then during the last 6 h, cells were pulsed with 0.5 μCi of tritiated thymidine and harvested, and cell-associated radioactivity counted as described for the cell proliferation assay. Half-maximal thymidine incorporation was considered as one unit and this was obtained with 1 pg of IL-6.

3. Results

3.1. Human fibroblast express Fas on the cell surface

Immunofluorescent analysis with specific monoclonal antibodies showed that Fas was expressed by normal fibroblasts (Fig. 1). These cells also expressed mainly p60 but very little p80 forms of the TNF receptors.

3.2. Fas antigen mediates proliferation of fibroblasts

Since TNF induces the proliferation of normal human foreskin diploid fibroblasts, we examined the effect of anti-Fas on these cells. The results shown in Fig. 2 indicate that, like TNF, anti-Fas induced the proliferation of fibroblasts in a dose-dependent manner. The effect of TNF appeared at concentrations as low as 0.1 ng/ml, whereas for anti-Fas, 10 ng/ml was required. Thus, the Fas antigen, like the TNF receptor, mediates not only cell killing but also cell proliferation. The proliferation of cells occurred even in the absence of the serum, but the overall increase was 4–10 times lower than of cells cultured with serum.

3.3. Fas-mediated fibroblast proliferation occurs by a mechanism different from that of TNF

Previously it has been reported that TNF-induced fibroblast proliferation can be completely blocked by orthovanadate, an inhibitor of protein tyrosine phosphatase [12]. We investigated whether orthovanadate also blocks anti-Fas-mediated proliferation of HDF. The results shown in Fig. 3 indicate that TNF-dependent proliferation was inhibited by orthovanadate but not that mediated through anti-Fas. This demonstrates that the mechanism of cell proliferation for anti-Fas differs from that of TNF.

3.4. Fas does not mediate IL-6 expression in fibroblasts

Since treatment of fibroblasts with TNF leads to the expression of IL-6, we examined the ability of anti-Fas to induce IL-6. Human fibroblasts were treated with 200 ng/ml of either TNF or anti-Fas for 24 h, and then the supernatants were assayed for IL-6 activity by bioassay. The results in Fig. 4 indicate that

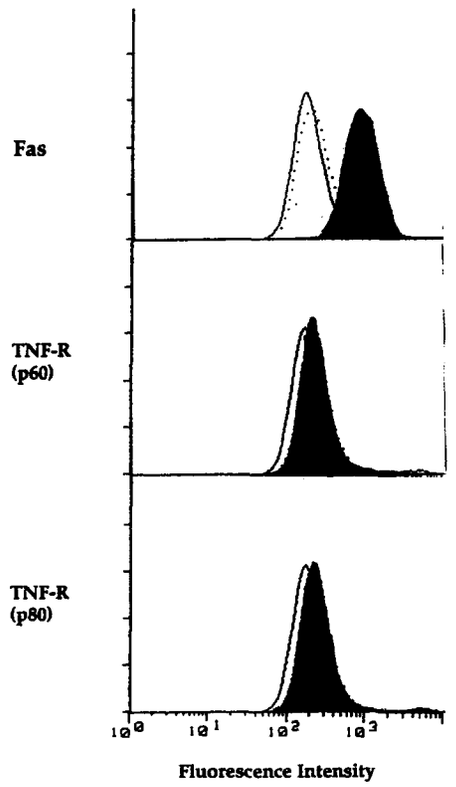

Fig. 1. Both Fas and TNF receptors are expressed on the surface of human fibroblasts. Cells (0.2×10^6/0.05 ml) were incubated with mcAb anti-Fas or anti-p60 (htr-9) or anti-p80 (utr-1) for 1 h on ice. After two washes, cells were incubated for an additional 1 h with fluorescein-conjugated goat anti-mouse IgM (for Fas) or goat anti-mouse IgG (for p60 and p80). Cells were washed, fixed and analyzed for fluorescence on a FACScan analyzer. Cells were stained by second antibody alone to determine background fluorescence. Unstained cells are shown as solid lines, cells stained with second antibody alone are dotted lines and those stained with first and second antibody are shown as shadded area.

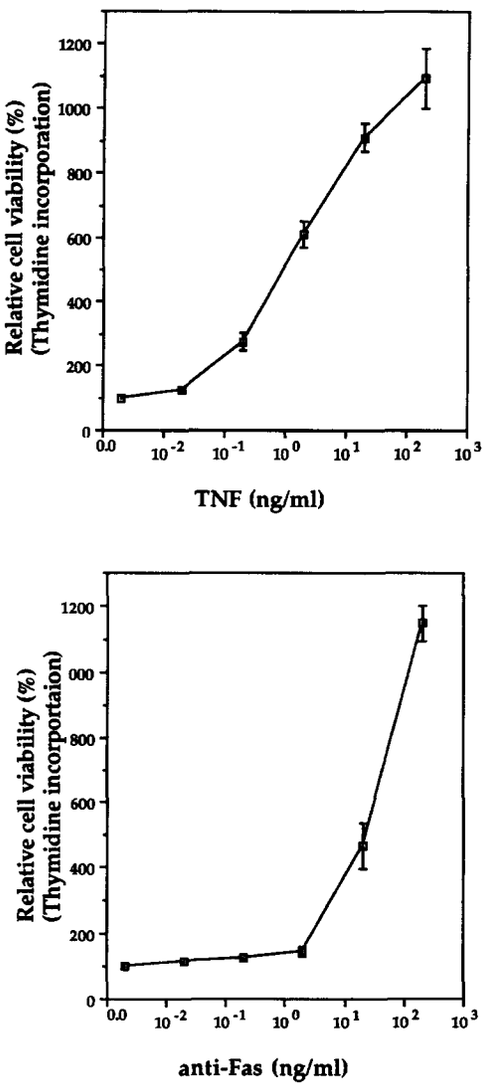

Fig. 2. Anti-Fas and TNF induces dose-dependent proliferation of normal diploid fibroblasts. Cells (8×10^3 in 0.1 ml) in 96-well plates were incubated with different concentrations of anti-Fas and TNF at 37°C for 72 h, and thereafter cell proliferation was determined by tritiated thymidine incorporation as described in section 2. All determinations were made in triplicate. The total mean cpm incorporation in the untreated control was 1196 (at 100% relative cell viability).

TNF was a potent inducer of IL-6 whereas anti-Fas has no effect. Thus even though HDF undergoes similar proliferative responses to both anti-Fas and TNF, their effect on IL-6 expression differs substantially.

4. Discussion

Both TNF and TNF receptor are structurally homologous to the Fas ligand and the Fas antigen, respectively. Whether Fas ligand (anti-Fas) and TNF have similar properties, however, is not known. In the present report we demonstrate that, like TNF, anti-Fas was found to induce the proliferation of normal human diploid fibroblasts, but by a mechanism different from that of TNF. Our results also show that, unlike TNF, anti-Fas did not induce the expression of interleukin-6.

Anti-Fas is a mAb that was made by immunizing mice with normal human diploid fibroblasts [6]. It was found to recognize a cell surface molecule, termed Fas, on different cell types and to induce cytotoxicity that could not be distinguished from that induced by TNF.

We found that anti-Fas does not just kill all the Fas-expressing cells; it also induces proliferation of normal fibroblasts. In this respect, anti-Fas displays a property similar to TNF. The proliferative effects of TNF on fibroblasts has been shown to be mediated through the p60 form of the TNF receptor [13] whose cytoplasmic domain exhibits homology to that of Fas. There has been no previous report of growth-stimulation by anti-Fas alone on normal cells but it has been shown that anti-Fas co-stimulates the proliferation of purified human T cells and thymocytes in the presence of anti-CD3 [14]. Interestingly, although both TNF and anti-Fas stimulated the proliferation of fibroblast, anti-Fas did not induce the expression of IL-6 in these cells but TNF did. Why anti-Fas would induce proliferation but not IL-6 expression is not clear, but there is clearly a difference in signaling between TNF and anti-Fas. This difference is further evident from our results that TNF-dependent proliferation of fibroblasts involves an orthovanadate-sensitive protein tyrosine phosphatase but not that mediated through anti-Fas.

It is not clear why TNF induces IL-6 expression in fibroblasts but anti-Fas does not. When TNF interacts with its receptor, one of the earliest events is the activation of a nuclear transcriptional factor, NF-κB, which could be observed within 5 min [15]. This activation leads to the induction of various genes including IL-6 [16]. In contrast, we found that interaction of

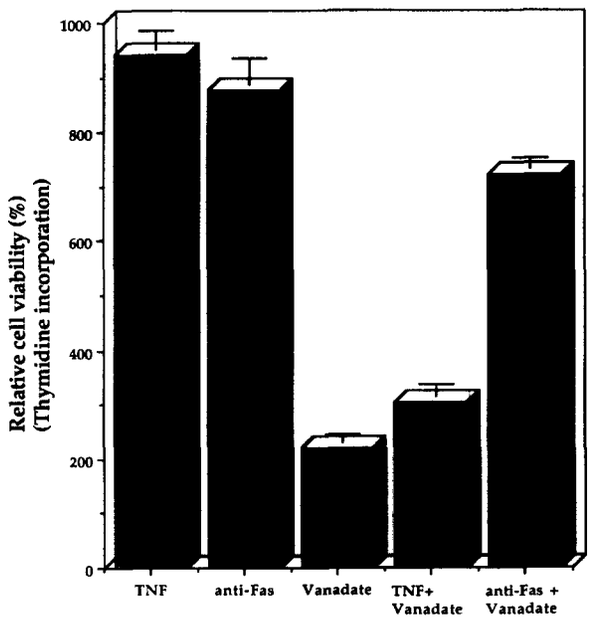

Fig. 3. Effect of orthovanadate on the anti-Fas- and TNF-dependent proliferation of normal diploid fibroblasts. Cells (8×10^3 in 0.1 ml) in 96-well plates were incubated with 100 ng/ml of either anti-Fas or TNF or orthovanadate (1 μg/ml) or indicated combinations at 37°C for 72 h. Thereafter cell proliferation was determined by tritiated thymidine incorporation as described in section 2. Results are based on proliferation of cells with media alone as 100%. All determinations were made in triplicate.

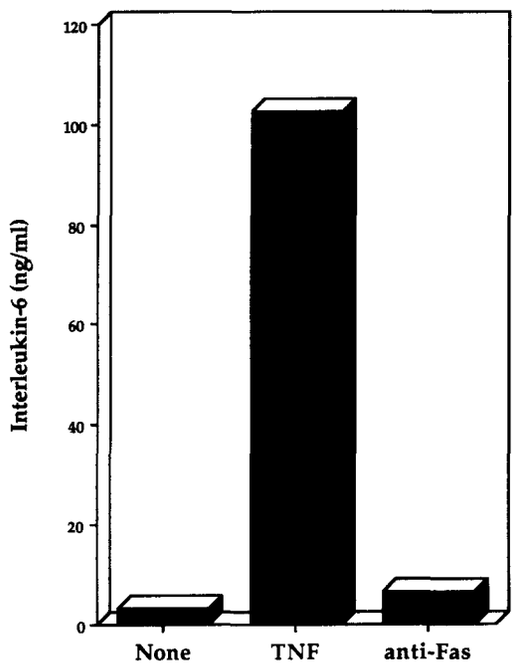

Fig. 4. Induction of expression of IL-6 by TNF and anti-Fas in fibroblast cells. 0.1×10^6 cells (1 ml) were incubated with TNF (200 ng/ml) or anti-Fas (200 ng/ml) at 37°C for 24 h and then cell supernatants were harvested for IL-6 determination by bioassay as described in section 2. All determinations were made in duplicate.

anti-Fas with its cell surface receptor did not activate NF-κB (data not shown), perhaps explaining why IL-6 expression was not induced by anti-Fas. The difference in the signaling of the p60 form of the TNF receptor and Fas has been reported previously [17]. Thus, overall our results indicate that anti-Fas exhibits not only cytotoxic but also growth-stimulatory effects that are similar to those of TNF, but the pattern of gene activation and its expression is quite different. The signals mediated through Fas and TNF receptor also seem to be independent of each other.

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