A CELL-KILLING MONOCLONAL ANTIBODY (ANTI-Fas) TO A CELL SURFACE ANTIGEN CO-DOWNREGULATED WITH THE RECEPTOR OF TUMOR NECROSIS FACTOR

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TNF was originally identified in the serum of mice treated sequentially with Bacillus Calmette-Guerin and endotoxin as a protein, causing hemorrhagic necrosis of some tumors in animals and producing cytolytic or cytostatic activity in some tumor cells in culture (1, 2). TNF is a monocyte/macrophage-derived protein exerting a variety of biological actions in addition to the cytotoxicity for tumor cells (2, 3). Recently, activated monocytes were reported to synthesize a transmembrane form of TNF on the cell surface and kill their target tumor cells by either cell-to-cell contact or local release of the TNF secretory component (4). TNF has been purified to homogeneity (5) and produced by recombinant DNA technology (6–9). As a result of these advances, highly purified rTNF has become available for experimental studies, and TNF has been shown to have multiple biological activities (2, 3). A variety of normal and transformed tumor cell lines have been shown to have high affinity cell surface receptors for TNF (10–14). No correlation was found between the susceptibility of cells to the cytotoxic activity of TNF and the quantity of TNF-Rs or the binding affinity of TNF to its specific receptor (11, 13). In this study, we describe an mAb to a human cell surface component (termed anti-Fas mAb) with associated cell-killing activity that is indistinguishable from the cytolytic activity of TNF. We suggest that the cytolytic activity of TNF is mediated by Fas antigen associated with TNF-R.

Materials and Methods

Cytokines and Cells. Purified human rTNF-α and purified human rIFN-γ were kindly provided by Fujisawa Pharmaceutical Inc., Osaka, and Dr. S. Nagata, Osaka Bioscience Institute, Osaka, respectively. Human rhabdomyosarcoma A673 and colon carcinoma HT29 were kindly provided by Dr. J. Vilček, New York University. Human foreskin-derived diploid fibroblast line TM11 was established in this laboratory.

Preparation of mAb. BALB/c female mice were immunized with the human diploid fibroblast FS-7 cell line and the spleen cells from immunized mice were fused with the NS-1 mouse myeloma line by standard hybridization technique (15). One hybridoma cell, producing an mAb with cell-killing activity (termed anti-Fas), was cloned two times by limiting dilution.

Anti-Fas mAb-producing hybridoma cells (2×10^8 cells) were cultured in 500 ml serum-free ASF104 medium (Ajinomoto Inc., Tokyo) for 5 d at 37°C. The culture fluid was concentrated by ultrafiltration using Omegacell (Filtron, Clinton), and was applied onto a hydroxylapatite column (Asahi Optical Inc., Tokyo). Anti-Fas mAb was eluted by 10–400 mM gradient

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of sodium phosphate, pH 7.4, by using FPLC system (Pharmacia Fine Chemicals, Uppsala). The eluted anti-Fas mAb was analyzed by SDS-PAGE and the purity was indicated to be >95%.

Assay of Cytolytic Activity. Cells were cultured in growth medium containing TNF or anti-Fas as indicated. Cells maintained in suspension culture were stained by trypan blue, and viable cells were quantified by phase-contrast microscopy. In monolayer cultures, the cytolytic activity was observed under phase-contrast microscopy, and then cell density was quantified by the staining method using amino black 10B as described by Vilček et al. (16).

Western Blotting. Plasma membrane fraction of U937 cell (300 μg protein/lane), prepared according to the method of Thom et al. (17), was subjected to 4–20% gradient PAGE in the presence of SDS. After electrophoresis, the proteins were transferred to a nitrocellulose sheet and Fas antigen on the nitrocellulose sheet was detected as follows. The nitrocellulose sheet was treated with 3% skim milk in PBS for 1 h at 37°C, and then washed by PBS containing 0.1% Tween 20 for 20 min at room temperature. Fas antigen on the nitrocellulose sheet was reacted with 20 μg/ml of purified anti-Fas IgM or 20 μg/ml of purified control monoclonal IgM antibody prepared in this laboratory, which did not bind to human cell surface. The reaction was visualized by an ABC kit (Vector Laboratories, Inc., Burlingame, CA).

Iodination and Binding Assay of TNF. Radioiodination of TNF using Iodogen (Pierce Chemical Co., Rockford, IL) was performed according to the method of Aggarwal et al. (18). The receptor binding assay with [^{125}I]TNF was performed as described previously in the case of IFN (19, 20). In brief, cells were incubated with [^{125}I]TNF in RPMI 1640 medium containing 10% FCS at 4°C for 5 h, and cell-bound radioactive TNF was measured.

Flow Cytometric Analysis. Cell surface Fas antigen and TNF-R were quantified as follows. Cells (5×10^5 cells/sample), washed with PBS, were reacted on ice for 1 h with 0.1 ml PBS containing 1% FCS, 0.02% NaN_3, 2 μg/ml biotin-TNF, and 20 μg/ml anti-Fas IgM, and then for an additional 1 h with 0.1 ml PBS containing 0.02% NaN_3, 100 μg/ml phycoerythrin-avidin D (Vector Laboratories, Inc.), and 10 μg/ml affinity-purified FITC goat anti-mouse IgM (Cappel Laboratories, Malvern, PA). After washing with PBS at 4°C, cell surface phycoerythrin-avidin and FITC anti-mouse IgM were quantified at the same time on a flow cytometer (Epics-CS).

Biotin-conjugated TNF was prepared by incubation of 100 μg TNF in 0.2 ml PBS with 300 μg sulfosuccinimidyl 6-(biotinamido) hexanoate (Pierce Chemical Co.) in 30 μl PBS for 20 min on ice, and then unconjugated biotin was removed by chromatography on a PD-10 column (Pharmacia Fine Chemicals).

Results

Comparison of the Cytolytic Activity of TNF and Anti-Fas mAb. Of >20,000 hybridoma clones producing mAbs to the human cell surface, one hybridoma produced an mAb that was cytolytic to various human cells. The mAb was shown to be IgM, and we termed it anti-Fas antibody. Anti-Fas IgM was purified to homogeneity, and electrophoretically pure anti-Fas IgM killed human cells as well as crude culture fluid of the hybridoma.

We compared the cell-killing activity of purified anti-Fas IgM and the cytolytic activity of human rTNF to human rhabdomyosarcoma A673. The cytolytic activity of TNF is well known to be enhanced when target cells are treated with IFN-γ, mitomycin C, actinomycin D, or cycloheximide. Fig. 1 shows that the cell-killing activity of TNF and anti-Fas is similarly enhanced when target cells are pretreated with IFN-γ or mitomycin C, or post-treated with actinomycin D or cycloheximide. Moreover, the cell-killing activity of TNF and anti-Fas was enhanced in the same way when target cells were incubated with the factors at high temperature (39°C).

In IFN-γ-treated A673 cells, purified anti-Fas IgM produced 50% and >90% cell killing at 10 ng/ml and 100 ng/ml (10 pM and 100 pM IgM), respectively. TNF killed IFN-γ-treated A673 cell to 50% and >90% at 2 ng/ml and 20 ng/ml (40 pM and

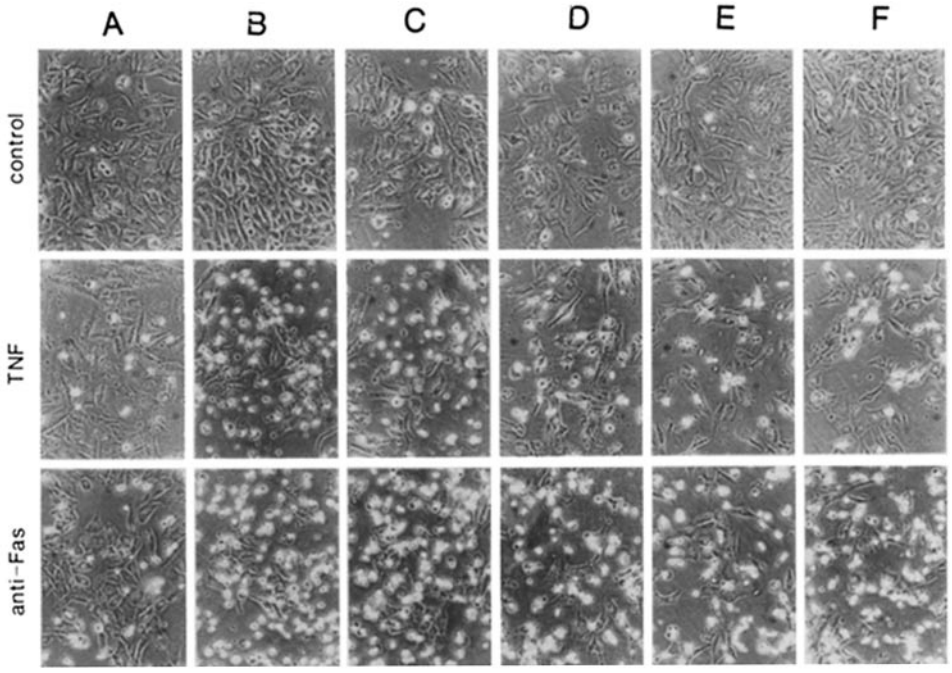

FIGURE 1. Cytolytic activity of TNF and anti-Fas IgM for the A673 rhabdomyosarcoma cell line. A673 cells were cultured with 10 ng/ml purified human rTNF or 30 ng/ml purified anti-Fas IgM for 15 h at 37°C, and cytolytic activity was observed under phase-contrast microscopy (*A*). Cells were pretreated for 24 h at 37°C with 200 IU/ml purified human rIFN-γ (*B*) or 0.08 μg/ml mitomycin C (*D*) before the treatment with TNF or anti-Fas IgM. Cells were incubated with TNF or anti-Fas at 39°C (*C*). Cells were cultured at 37°C for 4 h with 50 μg/ml cycloheximide (*E*) or 2.5 μg/ml actinomycin D (*F*) after the treatment with TNF or anti-Fas at 37°C.

400 pM TNF trimer), respectively. Thus, the molar concentrations of anti-Fas and TNF required to kill A673 cells are almost the same.

The cytolytic activity of TNF and purified anti-Fas IgM were also compared by time-lapse cinematography under a phase-contrast microscope (Fig. 2). Human A673 cells, pretreated with IFN-γ, were cultured with TNF or anti-Fas IgM at 38.5°C. Signs of cytotoxicity became detectable after 2 h of incubation with either TNF or anti-Fas IgM, with the number of killed cells increasing gradually thereafter. Almost all cells were killed by 15 h of incubation with either TNF or anti-Fas IgM.

Target Cell Specificity of TNF and Anti-Fas IgM. We analyzed the sensitivity to the cell-killing activity of anti-Fas IgM in several human cell lines that varied in their response to the cytolytic action of TNF (Table I). All human cell lines sensitive to the cytolytic activity of TNF were sensitive to anti-Fas IgM, and the cytolytic activity of both factors was similarly enhanced by the treatment with IFN-γ. It has been demonstrated that TNF and IFN-γ can exert a marked synergism in the cytotoxic activity on human colon carcinoma cell line HT29, which is almost insensitive to the cytolytic activity of TNF in the absence of IFN-γ (21). Our results show that HT29 cells are insensitive to the cytolytic activity of either TNF or anti-Fas, and pretreatment with IFN-γ renders HT29 cell sensitive to the cytolytic activity of both

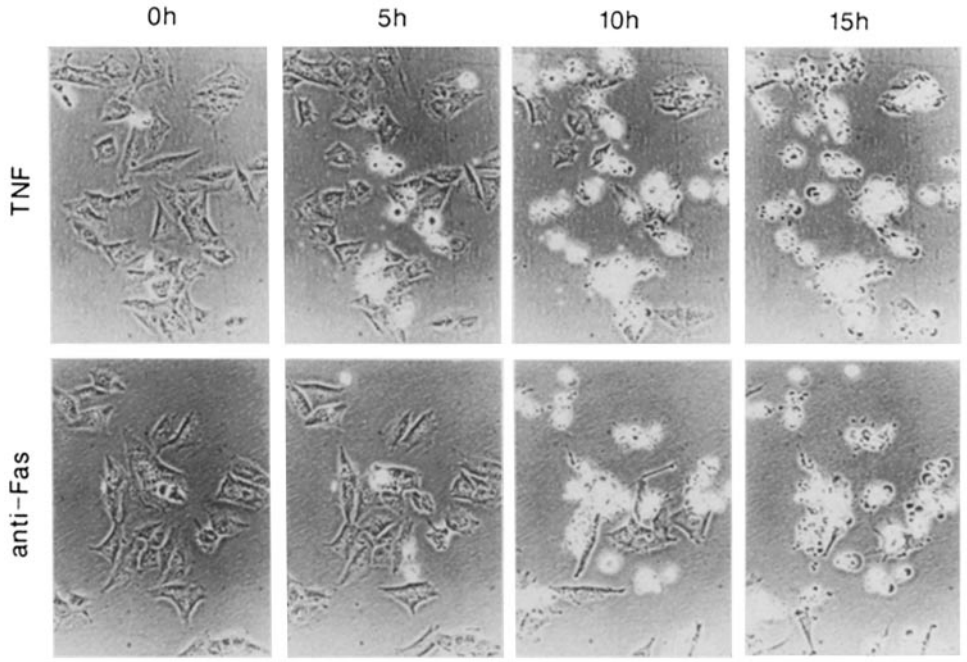

FIGURE 2. Comparison of the cytolytic activity of TNF and anti-Fas IgM. A673 cells pretreated with 200 IU/ml IFN-γ were incubated with 30 ng/ml TNF or 100 ng/ml purified anti-Fas IgM at 38.5°C, and the cytolytic activity of TNF and anti-Fas was monitored continuously by time-lapse cinematography under a phase-contrast microscope. Frames of the cinema after 0, 5, 10, and 15 h of incubation were photographed.

TABLE I

Target Cell Specificity of the Cytolytic Activity of TNF and Anti-Fas IgM

	Cytolytic effect			
	TNF		Anti-Fas IgM	
Cell	− IFN-γ	+ IFN-γ	− IFN-γ	+ IFN-γ
Histiocytoma U937	+ +	+ + + +	+ +	+ + + +
Promyelocytic leukemia HL60	+	+ + +	+	+ + + +
Rhabdomyosarcoma A673	+ +	+ + + +	+ +	+ + + +
Amnion-derived FL	−	+	+	+ + + +
Colon carcinoma HT-29	−	+ + + +	−	+ + + +
T lymphoblastoid MOLT4B	+	+	+	+
Diploid fibroblast FS-7	−	−	+	+ + +
Diploid fibroblast TM-11	−	−	+	+ + +
Burkitt lymphoma Daudi	−	−	−	−

Cells in growth medium (10^5 cells/ml) were cultured with or without 200 IU/ml IFN-γ for 24 h and then incubated with 20 ng/ml TNF or 50 ng/ml purified anti-Fas IgM for 15 h. The cytolytic activity was quantified as described in Materials and Methods. In the maximum response (+ + + +), 75–100% cells are killed; + + + denotes a strong response, that is, 50–75% cells killed; + +, a moderate response of 25–50% cells killed; +, a minimal response of <25% cells killed; and −, cells show no response to the cytolytic activity.

TNF or anti-Fas IgM. However, human diploid fibroblasts FS-7 and TM-11, which were insensitive to the cytolytic activity of TNF, were sensitive to the cell-killing activity of anti-Fas IgM. Burkitt lymphoma line Daudi was insensitive to both factors. We could not detect a significant number of TNF-Rs and Fas antigen on Daudi cells (data not shown).

Characterization of Fas Antigen by Western Blotting. Fas antigen on U937 cells was characterized by western blotting. Plasma membrane fraction of U937 cell was subjected to PAGE in the presence of SDS. After electrophoresis, the proteins were transferred to a nitrocellulose sheet and Fas antigen on the nitrocellulose sheet was analyzed. Fig. 3 shows a specific band with a molecular weight of 200,000 on the nitrocellulose filter treated with anti-Fas IgM. This band was not observed when control mouse IgM was used instead of anti-Fas IgM. Anti-Fas-specific band with molecular weight of 200,000 was also observed when SDS-PAGE was carried out in the presence of 2-ME. The molecular weight of Fas antigen is different from the molecular weight of the TNF-R, reported to be 65,000 ± 32,000 (22).

Downregulation of TNF-R on TNF-sensitive Cells by the Treatment with Anti-Fas IgM. Mo-

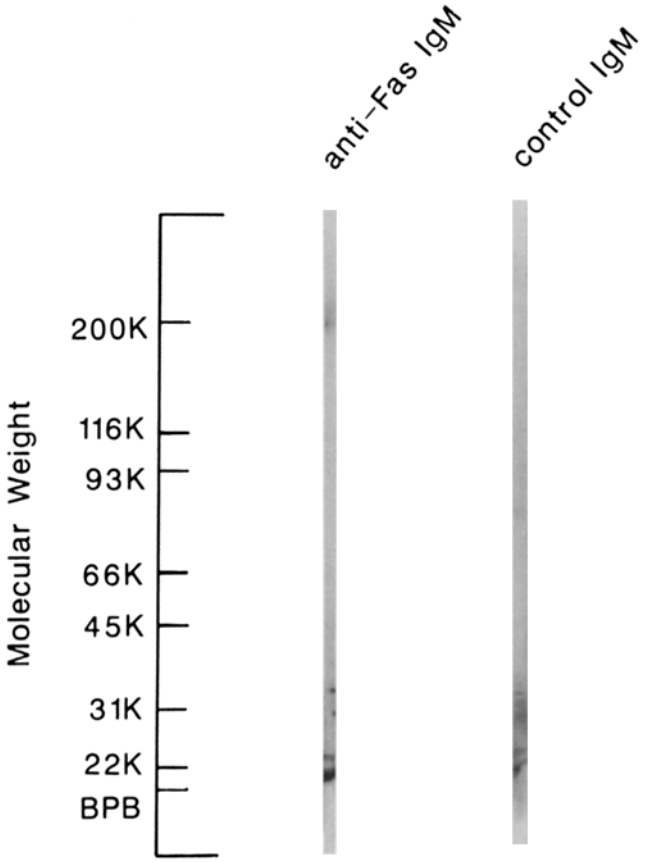

FIGURE 3. Western blot analysis of Fas antigen on U937 cell. Fas antigen on U937 cell was analyzed by western blotting as described in Materials and Methods. Molecular weight standards are shown on the left.

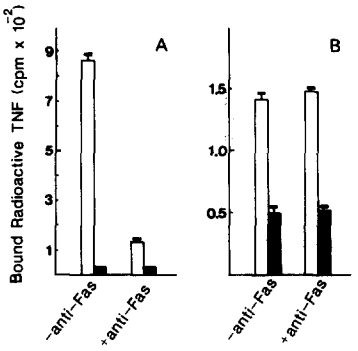

FIGURE 4. Radioactive TNF binding to U937 and TM11 cells pretreated with anti-Fas IgM at 37°C. U937 cells (*A*) and TM11 cells (*B*) were cultured in growth medium with or without 10 μg/ml purified anti-Fas IgM at 37°C. After a 5-h incubation, U937 cells (3.5×10^6 cells/ml) and TM11 cells (2.9×10^5 anti-Fas-treated cells/ml and 3.6×10^5 untreated cells/ml) were incubated with radioactive TNF (4,500 cpm and 0.62 ng/ml) in the presence (■) or in the absence (□) of unlabeled TNF (1 μg/ml). Radioactivity bound on cells was measured in duplicate as described in Materials and Methods.

lecular weight of Fas antigen was shown to be different from that of the TNF-R, although anti-Fas is similar to TNF in its cytolytic activity. To determine whether there is a relationship between the cytolytic activity of anti-Fas and TNF, we analyzed whether TNF-R was downregulated by the treatment with anti-Fas IgM. U937 and TM11 cells were incubated with 10 μg/ml of purified anti-Fas IgM at 37°C for 5 h, and then binding of radioactive TNF to cells was measured. In U937 cells, which are sensitive to the cytolytic activity of TNF, incubation with anti-Fas at 37°C did result in decreased binding of TNF (Fig. 4 *A*). On the other hand, incubation of TNF-insensitive TM11 cells with anti-Fas at 37°C did not result in decreased binding of TNF (Fig. 4 *B*). Scatchard analysis indicated that incubation of U937 cells with anti-Fas at 37°C for 5 h decreased the number of specific TNF binding sites to <20%, whereas incubation of TM11 cells with anti-Fas did not decrease the number of specific TNF binding sites (data not shown).

Co-downregulation of TNF-R and Fas Antigen by the Treatment with Either Anti-Fas or TNF. To show the co-downregulation of Fas antigen and TNF-R, we quantified simultaneously cell surface Fas antigen and TNF binding on a flow cytometer (Epics-CS) by using anti-Fas IgM and FITC-conjugated anti-mouse IgM, and biotin-conjugated TNF and phycoerythrin-conjugated avidin, respectively (Fig. 5). The binding of biotin-TNF to U937 cells was completely inhibited by a 100-fold excess of unlabeled TNF (Fig. 5 *A1*), indicating that the binding of biotin-TNF to U937 cell was specific.

We quantified both Fas antigen and TNF binding molecule on U937 cells pretreated with purified anti-Fas IgM (Fig. 5 *A*). Fas antigen and TNF binding on U937 cells, pretreated with anti-Fas IgM for 60 min on ice, were simultaneously downregulated by the incubation with anti-Fas at 37°C. After a 10-min incubation at 37°C, a portion of TNF-Rs was co-downregulated with Fas antigen, and after 60 min incubation at 37°C, almost all Fas antigen and TNF-Rs were co-downregulated.

We also quantified both TNF-R and Fas antigen on U937 cells, treated with biotin-conjugated TNF. U937 cells, pretreated with biotin-conjugated TNF for 60 min on ice, were incubated at 37°C for 10 min or 1 h in the presence of biotin-conjugated TNF, and then the amounts of cell surface Fas antigen and TNF binding were measured (Fig. 5 *B*). A portion of Fas antigen was co-downregulated with TNF binding after a 10-min incubation at 37°C. After a 1 h incubation at 37°C with TNF, about half of TNF binding and Fas antigen were co-downregulated.

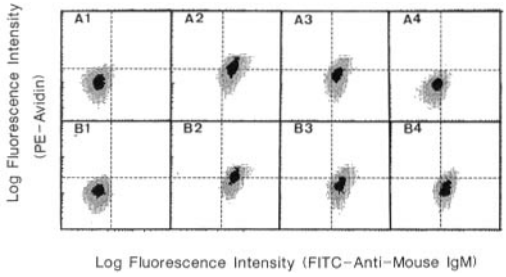

FIGURE 5. Co-downregulation of TNF-Rs and Fas antigen on human U937 cells treated with anti-Fas IgM or TNF. U937 cells were pretreated with 10 μg/ml anti-Fas IgM (*A*) or 2 μg/ml biotin-conjugated TNF (*B*) for 1 h on ice and then for 0 min (A2, B2), 10 min (A3, B3), or 60 min (A4, B4) at 37°C. Thereafter, cells were stained by anti-Fas IgM and biotin-conjugated TNF for 1 h on ice, followed by FITC anti-mouse IgM and phycoerythrin-avidin, and TNF-R and Fas antigen were quantified simultaneously on a flow cytometer. Cells were pretreated with control purified monoclonal IgM antibody instead of anti-Fas IgM and stained with control IgM and biotin-TNF in the presence of 100-fold excess of unlabeled TNF (*A1*). Cells were pretreated with unlabeled TNF instead of biotin-conjugated TNF and stained with control IgM and unlabeled TNF (*B1*).

We also analyzed whether Fas antigen was co-downregulated with the TNF-R on diploid fibroblasts TM11 and colon carcinoma HT29 cells, both of which were insensitive to the cytolytic activity of TNF. The quantity of Fas antigen was measured by means of indirect immunofluorescence on a flow cytometer before and after the incubation with TNF at 37°C. The amount of Fas antigen on neither cell decreased by the treatment with TNF (Fig. 6). TNF binding on either cell, however, was downregulated by the treatment with TNF (data not shown). Thus, Fas antigen and TNF binding molecules on TM11 and HT29 cells are not co-downregulated.

Since HT29 cell can be rendered sensitive to the cytolytic activity of TNF by the treatment with IFN-γ (Table I), we measured the amounts of Fas antigen on HT29 cells pretreated with IFN-γ (Fig. 6). The amount of Fas antigen on HT29 cells was

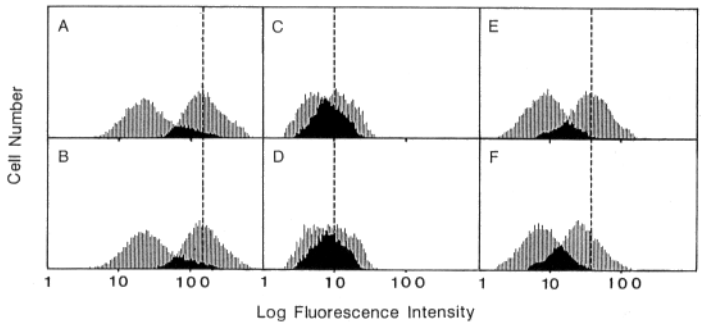

FIGURE 6. Flow cytometry analysis of Fas antigen on TM11 cell (*A* and *B*) and HT29 cell (*C–F*). Cells in 3.5-cm plastic dishes were incubated with 2 μg/ml TNF for 1 h on ice (*A*, *C*, and *E*) and then for 1 h at 37°C (*B*, *D*, and *F*). Thereafter, cells were detached from the culture dishes by a rubber policeman. And cells were reacted on ice for 1 h with 0.1 ml PBS containing 20 μg/ml anti-Fas IgM or 20 μg/ml control purified mouse IgM instead of anti-Fas, and then for an additional 1 h with 0.1 ml PBS containing 10 μg/ml affinity-purified FITC goat anti-mouse IgM. After washing with PBS at 4°C, cell surface FITC anti-mouse IgM were quantified on a flow cytometer (Epics-CS). HT29 cells were preincubated with (*E* and *F*) or without (*C* and *D*) 200 IU/ml IFN-γ for 15 h at 37°C before the incubation with TNF.

shown to markedly increase by the treatment with IFN-γ. Moreover, IFN-γ-treatment converted Fas antigen on HT29 cell to be downregulated by the treatment with TNF at 37°C.

Discussion

TNF is cytolytic or cytostatic for some types of tumor cells, and nontransformed cell lines are generally resistant to the cytolytic and cytostatic actions of TNF (2, 11, 13, 16, 21). There exists, however, indistinguishable TNF-R on tumor cells, sensitive to the cytolytic activity of TNF, and nontransformed cells (11, 13). It is unaccountable how to determine the sensitivity of cells to the cytolytic activity of TNF. We suggest here that the cytolytic activity of TNF is mediated by cell surface Fas antigen with a 200,000 mol wt associated with the TNF-R, and that TNF-R is not associated with Fas antigen on cells insensitive to the cytolytic activity of TNF.

In this study, we investigated an mAb anti-Fas IgM for human cell surface with associated cytolytic activity. The cytolytic activity of anti-Fas is indistinguishable from the cytolytic activity of TNF. The cytolytic activity of either anti-Fas or TNF was enhanced when target cells were treated with IFN-γ, metabolic inhibitors, and high temperature (Fig. 1). Moreover, we could not distinguish the cytolytic activity of anti-Fas from TNF when observed by the time-lapse cinematography under a phase-contrast microscope (Fig. 2). Anti-Fas IgM is shown to be agonistic mAb to TNF in the cytolytic activity.

Human cells sensitive to the cytolytic activity of TNF are sensitive to anti-Fas, but human cells sensitive to anti-Fas are not necessarily sensitive to the cytolytic activity of TNF (Table I). Human colon carcinoma HT29 is sensitive to the cytolytic activity of neither TNF nor anti-Fas, and IFN-γ treatment renders HT29 cells to be sensitive to both TNF and anti-Fas. Tsujimoto et al. (14) reported about twofold increase of TNF-Rs on HT29 cell by the treatment with IFN-γ. It seems to be difficult, however, to explain the conversion of HT29 cell by a twofold increase of TNF-Rs. In contrast, the amount of Fas antigen on HT29 cell is shown to increase exceedingly by the treatment with IFN-γ (Fig. 6). This marked increase of Fas antigen could be responsible for the conversion of HT29 cells in sensitivity to the cytolytic activity of anti-Fas as well as TNF.

Fas antigen was shown to be different from the TNF-R since (*a*) the molecular weight of Fas antigen (200,000) is different from the molecular weight of the TNF-R that was shown to be 65,000 ± 35,000 (22); and (*b*) the abundance of TNF-Rs on different cells does not correlate with the amounts of Fas antigen (data not shown). Thus, Fas antigen is not identical with the TNF-R, although anti-Fas is similar to TNF in the cytolytic activity.

Fas antigen on cells sensitive to the cytolytic activity of TNF seems to be associated with the TNF-R, because TNF-R and Fas antigen on sensitive cells are co-downregulated by the treatment with either anti-Fas or TNF (Fig. 5). In contrast, Fas antigen and TNF-R on cells insensitive to the cytolytic activity of TNF are not co-downregulated (Fig. 6). Human colon carcinoma HT29 was shown to be rendered sensitive to both TNF and anti-Fas by treatment with IFN-γ (Table I), and Fas antigen on IFN-γ-treated HT29 cell is shown to be downregulated by treatment with TNF (Fig. 6). There exists good correlation between the sensitivity to the cytolytic activity of

TNF and the codownregulation of Fas antigen and TNF-R. Thus, TNF-R may be associated with Fas antigen on cells sensitive to the cyolytic activity of TNF but is not associated with Fas antigen on TNF-insensitive cells.

It is well known that cellular macromolecular synthesis is not necessary to the cytolytic activity of TNF, although other activities of TNF may be caused by TNF-induced gene products. The cytolytic activity of TNF must be different from other activities of TNF. We analyzed whether other biological activities of TNF were associated with anti-Fas. Fibroblast growth-stimulating activity and HLA-inducing activity are not associated with anti-Fas (data not shown). Fas antigen seems to mediate only the cytolytic activity of TNF. Further studies on the purification and cDNA cloning of Fas antigen are in progress.

Summary

We have prepared an mAb specific for a human cell surface component (termed anti-Fas mAb). Anti-Fas shows cell-killing activity that is indistinguishable from the cytolytic activity of TNF. Fas antigen was characterized by western blotting, indicating that Fas antigen is a cell surface protein with a molecular weight of 200,000, which is different from the molecular weight of TNF-R. Fas antigen, however, is co-downregulated with the TNF-R when cells sensitive to the cytolytic activity of TNF are incubated with either TNF or anti-Fas. In contrast, Fas antigen on cells insensitive to TNF is not co-downregulated with the TNF-R. We suggest that the cell-killing activity of TNF is mediated by Fas antigen associated with the TNF-R.

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