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Mechanism of Fas-mediated cell death and its enhancement by TNF-α in human salivary gland adenocarcinoma cell line HSG

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Fas-mediated cell death in a human salivary gland adenocarcinoma cell line (HSG) was induced by treatment of the cells with agonistic anti-Fas antibody (CH-11), and this cell death was enhanced by pretreatment with tumor necrosis factor alpha (TNF-α). The mode of cell death was apoptosis, because it was accompanied by caspase activation and the cleavage of poly(ADP-ribose) polymerase. The TNF-α treatment of the cells increased the expression of Fas, which was accompanied by the activation of nuclear factor κB (NFκB). These results suggest that the enhancement of the apoptosis caused by TNF-α resulted from increased sensitivity of the HSG cells to CH-11-mediated apoptosis due to induction of Fas protein by TNF-α via the activation of NFκB. In order to elucidate the apoptosis signaling pathway, we examined the effect of various caspase inhibitors on the apoptosis induced by CH-11. Fas-mediated apoptosis of HSG cells was slightly inhibited by the caspase-9 inhibitor although it was mainly inhibited by that for caspase-8. Based on this finding, we consider CH-11-induced apoptosis in HSG cells to be mainly mediated by the type I death signaling pathway that is caused by a caspase cascade initiated by the activation of caspase-8 at the death-inducing signaling complex (DISC).

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The execution of apoptosis requires activation of a family of cysteine proteases called caspases (1, 2), which are ubiquitously and constitutively expressed as inactive zymogens (pro-caspases) in the cytosol (3–5). Caspases can be divided into two classes, 'initiator' (e.g. caspases-2, -8, -9, and -10) and 'effector' caspases (e.g. caspases-3, -6, and -7). The former class of caspases activate the latter, which are located downstream of the signal. In turn, these effector caspases degrade cellular substrates such as ICAD (inhibitor of caspase-activated DNase) and poly(ADP-ribose) polymerase (PARP), the enzyme that synthesizes poly(ADP-ribose) in response to DNA strand breaks and is involved in the maintenance of DNA integrity (6–8). Thus, this signal transduction results in cell death (3–5). The initiator caspases can be activated by either mitochondrion-mediated or cell-surface 'death' receptor-mediated processes (3–5). The mitochondrion-mediated process begins with the activation of initiator caspase-9, which then activates downstream effector caspases, such as caspase-3 and -6, thus leading to cell death (9–12). Activation of this caspase-9-dependent apoptosis pathway can be induced by many anticancer drugs (13). In contrast, the death receptor-mediated process activates an apoptotic pathway that is dependent on another initiator caspase, caspase-8 (14). In this pathway, caspase-3, -6 and/or -7 are mainly used as the downstream effectors.

The most studied death receptor is Fas (APO-1/CD95) (15). Fas is a 45-kDa transmembrane receptor that belongs to the tumor necrosis factor receptor (TNFR) superfamily (16–18). Cross-linking of the cell-surface Fas with the Fas ligand (FasL) or with agonistic anti-Fas antibody activates apoptotic death programs (18, 19). In the Fas signaling, when the FasL or agonistic anti-Fas antibody binds to the extracellular domain of Fas, the receptor becomes trimerized or oligomerized. This process is followed by the intracellular recruitment of the adapter protein FADD to the receptor cluster (14, 20–23); this event is facilitated by homotypic interactions between 'death domains' located in Fas and FADD. Next, the receptor-bound FADD oligomerizes, and then utilizes its second domain, the 'death effector domain', to recruit pro-caspase-8 (15, 20, 21). The complex consisting of Fas, FADD, and pro-caspase-8 is referred to as DISC (death-inducing signaling complex). In the DISC, proteolytic conversion of the pro-caspase-8 into active caspase-8 (14, 15) occurs. Caspase-8 then activates the downstream effector caspases, caspases-3, -6, and/or -7, the actions of which result in cell death. Moreover, it was reported that caspase-8 activated by the treatment of the cells with FasL or anti-Fas antibody could cleave Bid, which is one of the apoptosis-inducing members of the Bcl-2 family proteins, to cause the mitochondrion-mediated activation of caspase-9 (24–26).

The human salivary gland ductal cell line HSG, established from an irradiated human salivary gland, has characteristics of intercalated ductal epithelial cells (27), and it easily differentiates into various cell types in the presence of differentiation inducers such as 5-azacytidine (28) and retinoic acid (29). Therefore, we, and others, have used this cell line as a model system for salivary gland adenocarcinoma to study cell proliferation, differentiation, and apoptosis following treatment with different agents known to cause alterations in cellular status (28–30). Recently, it was reported that unstimulated HSG cells constitutively expressed low levels of Fas, that treatment of these cells with interferon gamma (IFN-γ) or tumor necrosis factor-alpha (TNF-α) consistently upregulated the level of Fas, and that the increased level of Fas contributed to the susceptibility of HSG cells to apoptosis induced by stimulation with agonistic anti-Fas antibody (31). However, the nature of the signaling pathways of Fas upregulation by those cytokines and agonistic anti-Fas antibody-triggered caspase activation in HSG cells have not yet been established.

A recent study revealed that apoptotic cell death occurs in salivary glands in patients with Sjögren syndrome, which is an organ-specific autoimmune disorder (32). This disease is presumably attributed to the loss of secretory function in salivary glands as a result of death of salivary cells in these glands (33–35). Several different cytokines originated from locally activated immune cells have been found in the salivary gland tissues of patients with Sjögren syndrome, among which TNF-α and IFN-γ play a pivotal role in the disease process of this syndrome (32).

The objective of the present study was to gain further insight into the mechanism of cell death induced in HSG cells following concomitant treatment with TNF-α and the agonistic anti-Fas antibody CH-11. We demonstrate herein that cell death induced following the treatment is caspase-dependent apoptosis, mainly mediated by the caspase-8–caspase-3 pathway at the initial stage. Moreover, the enhanced apoptosis induced by TNF-α was concluded to have probably occurred via TNF-α-mediated activation of nuclear factor κB (NFκB) and subsequent induction of Fas protein. Our findings may explain the molecular mechanism for the salivary gland cell death that occurs in patients with Sjögren syndrome.

Material and methods

Cell culture

Cells of HSG were cultured in Eagle's minimum essential medium (MEM) supplemented with 5% calf serum at 37°C in a humidified incubator with an atmosphere of 5% CO_2.

Determination of cell death

The HSG cells were seeded into 12-well dishes at an initial density of 5.0×10^5 cells per well. After cultivation for 2 d, the medium was changed; and the cells were then treated with 10 ng ml^{-1} recombinant human TNF-α (R & D Systems, Minneapolis, MN, USA), 100 ng ml^{-1} anti-human Fas monoclonal antibody clone CH-11 (Medical & Biological Laboratories, Nagoya, Japan) or the combination of TNF-α and CH-11 for various periods of time. Thereafter, the cells were stained with Trypan blue solution (final concentration of 2%), and cell death and survival were quantified by measuring the numbers of dye-stained and dye-excluding cells, respectively. Cell death was determined as a percentage of the total cell number.

Assay of caspase activity

The HSG cells were treated with 10 ng ml^{-1} TNF-α, 100 ng ml^{-1} CH-11, or a combination of them during short periods of incubation. The cells (1.0×10^6 cells) were then washed twice with ice-cold phosphate-buffered saline and lysed in 500 μl of 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)–KOH, pH 7.5, containing 20 mM sucrose, 50 mM KCl, 2.5 mM $MgCl_2$, and 1 mM dithiothreitol. The total cell lysates (100 μl) were assayed for their caspase activity by using 10 μl of 1 mM Ac-Asp-Glu-Val-Asp-NH-Mec (Peptide Institute, Osaka, Japan) as a substrate. After incubation at 37°C for 30 min, the enzyme reaction was terminated by adding 100 μl of 50% (v/v) acetic acid. The amount of 7-amino-4-methylcoumarin liberated was determined as described by Kojima *et al.* (36). One unit of caspase activity was defined as the amount that catalyses the liberation of 1 mmol of 7-amino-4-methylcoumarin min^{-1} mg^{-1} protein under the experimental conditions used.

Reverse transcriptase polymerase chain reaction (RT-PCR)

The HSG cells were treated with 10 ng ml^{-1} TNF-α for various periods of time. Total RNA was isolated with ISOGEN reagent (Nippongene, Tokyo, Japan) according to the manufacturer's instructions. First-strand cDNA was synthesized from total RNA by use of TrueScript II and the supplied Random 9 mer primer (Sawady Technology, Tokyo, Japan). Reverse transcription was performed by using a thermal program of 42°C for 60 min and 70°C for 15 min. Polymerase chain reaction was performed with the following primers: 5′-CTGCCTCAATGGGACCGTGC-3′ and 5′-CGGGGGTATAGGTGGAGCTG-3′ for TNFR-I; 5′-TCTAACTTGGGGTGGCTTTGTCTTC-3′ and 5′-GTGT CATACGCTTCTTTCTTTCCAT-3′ for Fas; 5′-CCAGT CGCTTTGTGCCATGC-3′ and 5′-GCCTTTGACCATGC CCACAG-3′ for caspase-3; 5′-GATGCAGGGGCTTTGA CCAC-3′ and 5′-CCCACCAGAAAGTCAGCCTC-3′ for caspase-8; 5′-CGGTGCTCTGGACTGCTGCG-3′ and 5′-GCAACGGGGTGGCATCTGGC-3′ for caspase-9; and 5′-TGGTATCGTGGAAGGACTCATG-3′ and 5′-TCTCT TCCTCTTGAGCTCTTGC-3′ for glyceraldehyde adenosine-phosphate dehydrogenase (GAPDH). Products of 460, 340, 630, 409, 286, and 549 bp, respectively, were obtained. Conditions for the 20 cycles of PCR were 95°C for 30 s, 60°C for 60 s, and 72°C for 90 s. Samples were resolved on a 2% agarose gel and visualized with ethidium bromide. All bands were quantified by densitometry using NIH Image (National Institutes of Health, Bethesda, MD, USA). Values for TNFR-I, Fas and the three caspases were normalized to those obtained for the corresponding GAPDH. The relative expression level was shown as the fold increase or decrease of each band based on the 'control'.

Preparation of nuclear fraction

The HSG cells were seeded into 90-mm dishes at an initial density of 1.5×10^6 cells per dish. After cultivation for 5 d, the medium was changed; and the cells were then treated with 10 ng ml^{-1} TNF-α for various periods of time. Next, the cells were homogenized in 500 μl of 10 mM Tris-HCl, pH 7.4, containing 1 M hexylene glycol, 0.5 mM $MgCl_2$, 1 mM ethyleneglycoltetraacetic acid (EGTA), 2 mM leupeptin, and 0.01% aprotinin by passing them 20 times through a 25-gauge needle attached to a plastic syringe. The homogenate was centrifuged at 1500 ***g*** for 10 min, and the nuclear pellet was suspended in 500 μl of the same buffer and centrifuged again under identical conditions. After centrifugation, the nuclear pellet was treated with 100 μl of 10 mM Tris-HCl, pH 7.4, containing 0.3 M KCl, 10% glycerol, 1 mM dithiothreitol, 2 mM leupeptin, and 0.01% aprotinin for 30 min at 0°C. The nuclear extract obtained following centrifugation at 10 000 ***g*** for 1 h was mixed with 100 μl Laemmli buffer containing β-mercaptoethanol and boiled at 100°C for 10 min. Twenty microliters of the extract was analysed by Western blotting with anti-NFκB (p65) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Apoptosis-neutralizing activity of ZB4 clone of anti-Fas antibody

The HSG cells were treated for 1 h with various concentrations of antagonistic antihuman Fas monoclonal antibody (clone ZB4; Medical & Biological Laboratories) in fresh medium. Apoptosis was triggered by the addition of 100 ng ml^{-1} CH-11. After 24 h, cell viability was determined by the Trypan blue-exclusion method described above. Details of the treatments are described in the appropriate figure legends.

Effect of caspase inhibitors

The HSG cells were pretreated with 10 ng ml^{-1} TNF-α for 6 h. The cells were then washed once with 1 ml of medium and treated with a 20 μM concentration of each of various caspase inhibitors in fresh medium. The following caspase inhibitors were used in this study (all caspase inhibitors were obtained from Medical & Biological Laboratories): Z-Asp-Glu-Val-Asp-fmk for caspase-3; Z-Ile-Glu-Thr-Asp-fmk for caspase-8; Z-Leu-Glu-His-Asp-fmk for caspase-9; and Z-Val-Ala-Asp-fmk for pan-caspases. Two hours later, 100 ng ml^{-1} CH-11 was added, and the cells were further incubated for 20 h. Cell viability was determined by using the Trypan blue-exclusion method as described above.

Western blot analysis

HSG cells were seeded into 12-well dishes at an initial density of 5.0×10^5 cells per well. After cultivation for 2 d, the medium was discarded; and the cells were then treated with 10 ng ml^{-1} TNF-α, or 100 ng ml^{-1} CH-11, or a combination of them for various periods of time. When used, SN-50 (10 μM), which is the inhibitor for NFκB, was added to the medium 1 h prior to the addition of TNF-α. Total cell lysates were obtained by heating the cells (1.0×10^6) at 100°C for 10 min in 100 μl Laemmli buffer containing β-mercaptoethanol. Equal amounts of proteins were separated on a 5–20% acrylamide gradient gel containing sodium dodecyl sulphate (SDS). Proteins were then transferred electrophoretically to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA, USA). Specific proteins were detected by using a VECTASTAIN ABC-AmP Western blotting immunodetection kit (Vector Laboratories, Burlingame, CA, USA) or an ECL Plus Western blotting detection system (Amersham Biosciences, Piscataway, NJ, USA). The following primary antibodies were used: anti-PARP (Santa Cruz Biotechnology), anti-Fas (clone ZB4; Medical & Biological Laboratories), and anti-IκB (Upstate Biotechnology, Lake Placid, NY, USA).

Statistics

Data were presented as the mean ± standard deviation (SD). Statistical analysis was performed by using Student's *t*-test, and values of $P < 0.05$ were considered to be significant.

Results

Agonistic anti-Fas antibody-induced apoptosis in HSG cells

Agonistic anti-Fas antibody (clone CH-11), a mouse monoclonal antibody (IgM) specific for an epitope on the extracellular domain of human Fas, has been shown to induce the apoptosis of various kinds of cells. Therefore, in this study, HSG cells were treated, or not, with TNF-α alone, CH-11 alone, or a combination of the pretreatment with TNF-α and subsequent incubation with CH-11. In the time-course analysis, we observed a decrease in cell viability at 12 h when the cells were given the combination treatment (Fig. 1A, circles). The proportion of dead cells was approximately 20% at 12 h and increased to 60% at 24 h, indicating that cell death occurred in a time-dependent manner. The percentage of cell death was higher in the cultures that had been treated with both TNF-α and CH-11 than in those incubated with CH-11 alone (Fig. 1A, squares). However, as in the control, no significant cell death was found when the cells were treated with TNF-α alone (Fig. 1A, diamonds). When the caspase activity was measured by using a substrate specific for caspase-3, as in the case of cell death, the combination treatment resulted in a higher activity than the treatment with CH-11 alone (Fig. 1B). In both cases, the activity was increased time dependently, starting 1–3 h after the addition of CH-11. However, no caspase activity was detected in cultures treated with TNF-α alone. In order to determine whether the HSG cells were killed via apoptotic processes, we had previously examined internucleosomal DNA fragmentation, one of the well-known biochemical characteristics of apoptotic cells. When HSG cells were treated with TNF-α and then with CH-11, apoptotic DNA laddering appeared slightly at 12 h, and was clearly visible at 24 h after the treatment with CH-11. In the present experiment, as described above, the time-dependent activation of caspase preceded the cell death (Fig. 1A,B). Moreover, the cleavage of PARP, a biochemical hallmark of apoptosis, was observed in HSG cells treated with CH-11 alone or with TNF-α and subsequently CH-11 (Fig. 1C).

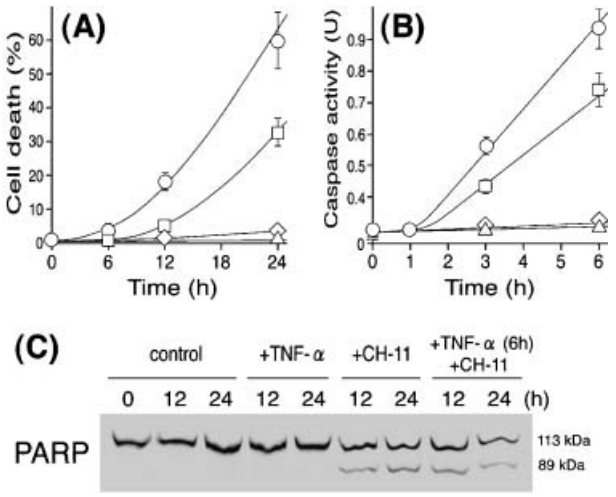

Fig. 1. Time-course analysis of cell death (A), caspase activity (B), and cleavage of poly(ADP-ribose) polymerase (PARP) (C). Human salivary gland adenocarcinoma cell line (HSG) cells were seeded into 12-well dishes at an initial density of 5.0×10^5 cells per well. After cultivation for 2 d, the cells were treated with 10 ng ml^{-1} tumor necrosis factor alpha (TNF-α) alone (diamonds), 100 ng ml^{-1} agonistic anti-Fas antibody (CH-11) alone (squares) or left untreated (triangles) for the indicated times. For the combination group, cells were pretreated with 10 ng ml^{-1} TNF-α for 6 h and then treated with 100 ng ml^{-1} CH-11 for the indicated times (circles). *(A)* Cells were stained with Trypan blue solution, and cell death was quantified by measuring the number of dye-stained cells (values are mean ± SD, $n = 5$). *(B)* The caspase activity was routinely assayed with Ac-Asp-Glu-Val-Asp-NH-Mec as a substrate. One unit of caspase activity was defined as the amount that catalyses the liberation of 1 mmol of 7-amino-4-methylcoumarin min^{-1} mg^{-1} protein (data are mean ± SD, $n = 5$). *(C)* Total cell lysates were prepared by harvesting the cells in 100 μl of Laemmli buffer containing β-mercaptoethanol and heating them at 100°C for 10 min. Samples were analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 5–20% acrylamide gradient gels, and Western blotting for PARP was then performed. Results shown are representative of those obtained in four additional experiments.

Our results thus demonstrate that the cell death triggered by CH-11 in HSG cells was indeed apoptosis.

Expression levels of apoptosis-related genes

The HSG cells were treated with TNF-α for various periods. Total RNA was extracted from these cells, and the expressions of various apoptosis-related genes (TNFR-I, Fas, caspase-3, caspase-8, and caspase-9) were analysed by RT-PCR; GAPDH was included as an internal control. As shown in Fig. 2, the mRNA expression levels of Fas and caspases were changed by the treatment. The level of Fas mRNA increased 1–3 h (approx. five- to ten-fold) after the start of treatment with TNF-α and then decreased by 6 h. This observation indicates that TNF-α upregulated the expression of Fas mRNA. As regards expression levels of caspase mRNAs, the TNF-α treatment increased those of caspase-3 and caspase-8, and the maximum level was seen at 3 h in both cases. The inductive effect was more prominent for

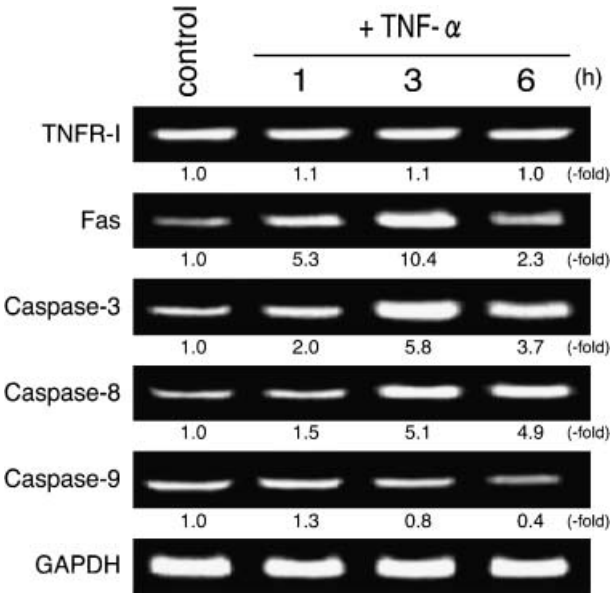

Fig. 2. Expression of apoptosis-relating genes. Human salivary gland adenocarcinoma cell line (HSG) cells were seeded into 12-well dishes at an initial density of 5.0×10^5 cells per well. After cultivation for 2 d, the cells were treated with 10 ng ml^{-1} tumor necrosis factor alpha (TNF-α). After the indicated periods of incubation, total RNA was extracted from these cells, and the mRNA expressions of TNFR-I, Fas, caspase-3, caspase-8, and caspase-9 were analysed by reverse transcriptase polymerase chain reaction. Samples were resolved on a 2% agarose gel and visualized with ethidium bromide. All bands were quantified by densitometry using NIH Image. Values for tumor necrosis factor receptor (TNFR)-I, Fas and the three caspases were normalized to those obtained for the corresponding glyceraldehyde adenosine-phosphate dehydrogenase (GAPDH). The relative expression level is shown as the fold increase or decrease of each band based on the 'control.' Results shown are representative of data obtained in four additional experiments.

caspase-3 than for caspase-8. However, the level of caspase-9 mRNA was unexpectedly decreased after the treatment with TNF-α. The reason for this downregulation of caspase-9 is unknown and remains to be clarified. The mRNA expression level of TNFR-I was not changed by the stimulation with TNF-α. FasL was not detected irrespective of treatment with TNF-α (unpublished data).

Upregulation of Fas protein level by TNF-α treatment

The HSG cells were treated with TNF-α for various periods. Total cell extracts were used for Western blotting of Fas and inhibitor of κB (IκB), whereas nuclear extracts were prepared and used for that of NFκB (p65). As shown in Fig. 3A, the expression level of Fas protein was increased by the TNF-α treatment. The increase in level was detected after only 1 h of treatment (4.9-fold), and the maximum expression (16.4-fold) was found at 12 h. This result supports the data shown in Fig. 2. Namely, the induction of Fas mRNA could cause the subsequent induction of its cognate protein Fas. To examine whether the expression of Fas was a consequence of NFκB activation, we used SN-50, a

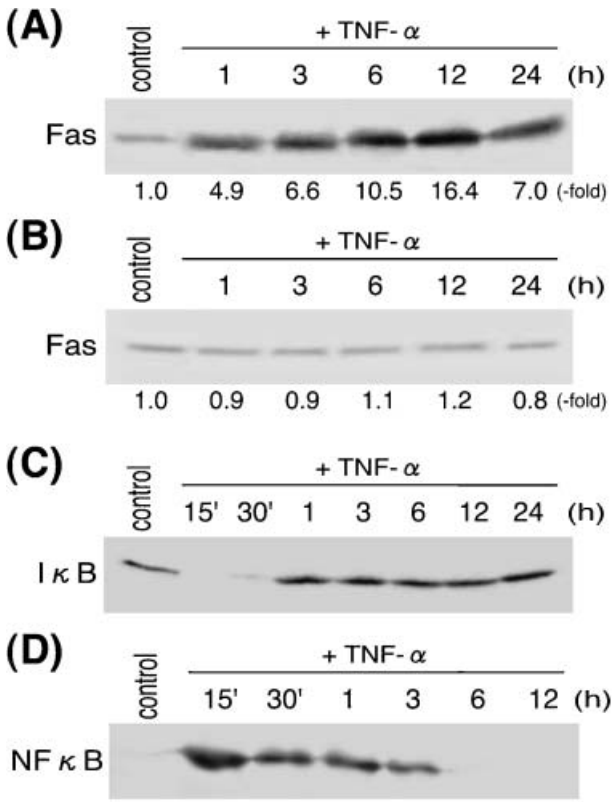

Fig. 3. Western blot analysis of Fas expression, inhibitor of κB (IκB) degradation, and nuclear translocation of nuclear factor κB (NFκB) after treatment with tumor necrosis factor alpha (TNF-α). Human salivary gland adenocarcinoma cell line (HSG) cells were seeded into 90-mm dishes at an initial density of 1.5×10^6 cells per dish. After cultivation for 5 d, the medium was changed; and the cells were then treated with 10 ng ml^{-1} TNF-α for the indicated periods of time. When used, SN-50 (10 μM) was added to the medium 1 h prior to the addition of TNF-α. Fas expression *(A)*, Fas expression after SN-50 (inhibitor for NFκB) pretreatment *(B)*, IκB degradation *(C)*, and nuclear translocation of NFκB *(D)* were analysed by Western blot using total cell lysates (A–C) or nuclear extracts (D). The bands in (A) and (B) were quantified by densitometry using NIH Image. The relative expression level is shown as the fold increase or decrease of each band based on the 'control.' Results shown are representative of data obtained from four additional experiments. Details are given in the Material and methods section.

cell-permeable peptide that inhibits NFκB by blocking its translocation to the nucleus (37). Previous studies have demonstrated that SN-50 is highly selective against NFκB and has no effect on the activities of any other signaling molecules (38). Treatment of HSG cells with SN-50 greatly reduced TNF-α-inducible Fas expression (Fig. 3B). Moreover, as shown in Fig. 3C and D, IκB was degraded and disappeared from the cells in less than 15 min; concomitantly, NFκB appeared in the nucleus and was retained there for 3 h when the cells were treated with TNF-α. IκB appeared again within 1 h. Using immunocytochemical staining we confirmed the translocation of NFκB to the nucleus (data not shown). These results indicate that NFκB was activated by treatment with TNF-α, strongly suggesting that the upregulation of Fas by TNF-α occurred through the induction of Fas protein mediated by NFκB signaling.

Neutralizing effect of antagonistic anti-Fas antibody (clone ZB4) on the apoptosis induced by CH-11 in HSG cells

To investigate whether the CH-11-induced apoptosis was dependent on the binding between CH-11 and Fas on the cell surface, we conducted a competition assay by using antagonistic antibody ZB4. This binds to Fas on the cell surface and competitively inhibits the cell death signaling downstream of Fas (16,39). As shown in Fig. 4, the CH-11-induced apoptosis of HSG cells that had not been treated with TNF-α was inhibited dose-dependently by pretreatment with various concentrations of ZB4, and the inhibition was almost complete at the dose of 10 μg ml^{-1} (Fig. 4, triangles). When various concentrations of ZB4 were added to the culture medium after the cells have been pretreated with TNF-α for 3 h, the

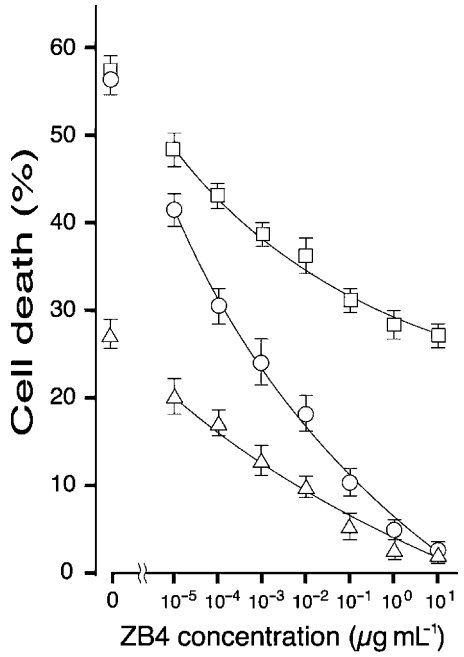

Fig. 4. Neutralizing activity of anti-Fas antibody-induced apoptosis by ZB4 clone. Human salivary gland adenocarcinoma cell line (HSG) cells were seeded into 12-well dishes at an initial density of 5.0×10^5 cells per well. After cultivation for 2 d, the cells were treated as follows: circles, 10 ng ml^{-1} tumor necrosis factor alpha (TNF-α) for 3 h, a wash with medium, and then incubation with the indicated concentrations of ZB4 for 1 h; squares, indicated concentrations of ZB4 for 1 h, wash with medium, and then 10 ng ml^{-1} TNF-α for 3 h; triangles, indicated concentrations of ZB4 for 1 h. After the cells had been washed with phosphate-buffered saline, 100 ng ml^{-1} CH-11 was then added to the cultures, which were further incubated for 24 h (data are means ± SD, $n = 5$). Cell viability was determined by the Trypan blue-exclusion method described in the Material and methods section. Results shown are representative of data obtained from four additional experiments.

enhanced apoptosis induced by treatments with TNF-α and subsequent CH-11 treatment was inhibited dose-dependently and almost completely at a ZB4 dose of 10 μg ml^{-1} (Fig. 4, circles), as in the case of the TNF-α-untreated HSG cells (Fig. 4, triangles). In contrast, when the cells were first treated with ZB4 for 1 h, with TNF-α for the following 3 h after removal of ZB4, and then with CH-11, the enhanced apoptosis was not completely inhibited by even as high a concentration of ZB4 as 10 μg ml^{-1}; although a mild dose-dependent inhibition was also shown over the range of ZB4 tested (Fig. 4, squares). Approximately 25–30% of the cells were still dead in the presence of 10 μg ml^{-1} of ZB4. This percentage of dead cells is consistent with that for TNF-α-untreated, CH-11-treated cells in the absence of ZB4 (Fig. 4, triangles). These results thus strongly suggest that the death of those 25–30% cells (Fig. 4, squares) occurred through binding between CH-11 and Fas that had been newly synthesized in response to TNF-α treatment after the ZB4 had been washed out.

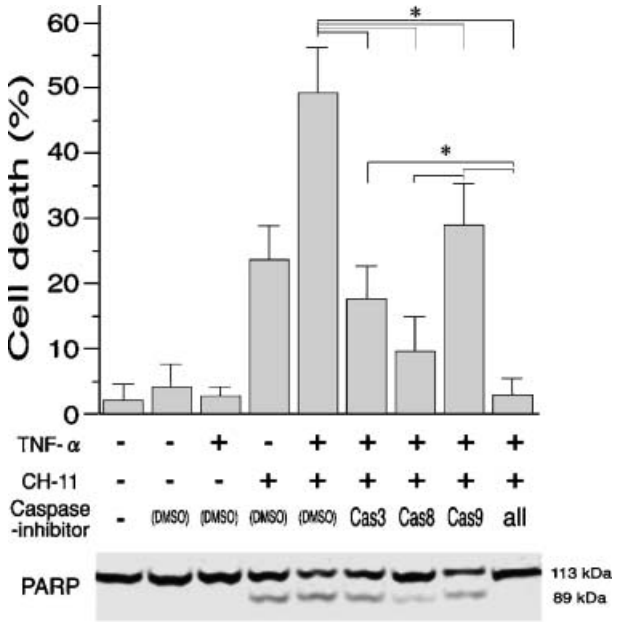

Fig. 5. Effect of various caspase inhibitors on cell death. Human salivary gland adenocarcinoma cell line (HSG) cells were seeded into 12-well dishes at an initial density of 5.0×10^5 cells per well. After cultivation for 2 d, the cells were pretreated, or not, with tumor necrosis factor alpha (TNF-α) for 6 h, and then treated or not with a 20 μM concentration of each of various caspase inhibitors for 2 h. The following caspase inhibitors were used: Z-Asp-Glu-Val-Asp-fmk for caspase-3 (*Cas3*); Z-Ile-Glu-Thr-Asp-fmk for caspase-8 (*Cas8*); Z-Leu-Glu-His-Asp-fmk for caspase-9 (*Cas9*); and Z-Val-Ala-Asp-fmk for pan-caspases (*all*). After the treatment with a caspase inhibitor, the cells were stimulated with agonistic anti-Fas antibody (CH-11) for 20 h. The cell death was measured by the Trypan blue-exclusion method as described in the Material and methods section (upper panel); the cleavage of poly(ADP-ribose) polymerase (PARP) was analysed by Western blot analysis (lower panel). Results shown are representative of data obtained from four additional experiments (data are means ± SD, $n = 5$). *, $P < 0.05$, from *t*-test.

Effect of caspase inhibitors on the apoptosis induced in HSG cells

The HSG cells were pretreated with TNF-α for 6 h, and then with various caspase inhibitors for 2 h. After the treatment with a given caspase inhibitor, the cells were stimulated with CH-11 for 20 h, and then cell death was assessed. As shown in Fig. 5 (upper panel), compared with the cells treated with TNF-α and CH-11, and without any caspase inhibitors, apoptosis was significantly inhibited by the treatment with the specific inhibitors for caspases-3, -8 and -9 ($P < 0.05$ from *t*-test). The apoptosis was almost completely blocked by use of a pan-caspase inhibitor, which inhibits all kinds of caspases ($P < 0.05$ from *t*-test, indicated in the figure as 'all'). Western blot analysis, as shown in Fig. 5 (lower panel), revealed that the cleavage of PARP was partly inhibited by the treatment with caspase inhibitors (the level of PARP cleavage corresponded with that of the cell death shown in the upper panel). The inhibitor for pan-caspases ('all') completely blocked the cleavage of PARP, resulting in the 113 kDa-band intensity similar to that of untreated, solvent (DMSO)-treated control or to that obtained with TNF-α treatment alone. This result also supports the finding shown in the upper panel of Fig. 5.

Discussion

The present study showed that the pretreatment of HSG cells with TNF-α enhanced the apoptosis that was triggered by CH-11 by increasing the expression of Fas via the activation of NFκB. In our experiment, however, apoptosis was not induced by the treatment with TNF-α alone (Fig. 1A). The recent study by Kamachi *et al.* (40) showed that the treatment of HSG cells with TNF-α alone or INF-γ alone for a prolonged period, such as 96 h, induced apoptotic cell death. In that experiment, TNF-α was shown to cause the apoptosis through a caspase-8-dependent pathway. In our experiment, however, the treatment with TNF-α alone did not cause the apoptosis by 24 h. The difference between their data and ours was probably due to the treatment period. The treatment period of our experiment was limited up to 24 h. If the period of the TNF-α treatment had been prolonged, however, the percentage cell death probably would have increased to a level similar to that reported in the above study. Therefore, in our study, there is not a problem because we were focusing on the short-term effect of TNF-α. Our results are consistent with previously reported findings, in which Fas-mediated apoptosis of salivary gland cells was enhanced by pretreatment of the cells with TNF-α or IFN-γ (31,41,42). Generally, Fas-mediated apoptosis is regulated in part by the levels of expression of Fas and FasL (43–45). In an experiment conducted earlier, however, the mRNA level of FasL did not change (unpublished data), although that of Fas did. The data shown in Fig. 2 suggest that the increased mRNA expressions of Fas, caspase-3, and caspase-8 by the TNF-α pretreatment enhanced the sensitivity for cell death triggered by CH-11 in HSG cells.

In the process of TNF-α signaling, occupation of TNFR by TNF-α triggers both caspase-dependent and caspase-independent signaling cascades. The caspase-dependent signaling pathway induces apoptotic cell death in susceptible cells, whereas the caspase-independent signaling cascade leads to activation of NFκB and induces anti-apoptotic or proliferative activities (46–48). In the NFκB activation process, stimulation by a cytokine such as TNF-α activates, via receptor interacting protein (RIP), IκB kinase. In turn, this kinase phosphorylates IκB, resulting in release of IκB from the NFκB and degradation of the former via proteasome-dependent proteolysis. Once IκB is released, NFκB is immediately translocated into the nucleus, where it regulates gene expression. However, by promoter analysis of the Fas gene, the NFκB binding site was found in the 5′-upstream region and was revealed to be biologically functional during cytokine stimulation, oxidative stress, or chemotherapy-induced apoptosis in distinct cell types (49–53). Considering these findings, we thought it likely that the induction of the Fas gene by TNF-α in HSG cells would be mediated through the activation of NFκB. From the activation of NFκB by the release of IκB and the translocation of NFκB to the nucleus after the stimulation of the HSG cells with TNF-α, the enhanced expression of the Fas gene would seem to be mediated by the NFκB signaling pathway, as shown in Fig. 3.

In order to identify the apoptosis signaling pathway, we examined the effect of various caspase inhibitors on the apoptosis induced by CH-11. Two different signaling pathways downstream of Fas, types I and II, have been proposed (54). In type I signaling, the death signal is transduced by a caspase cascade initiated by the activation of caspase-8 at the DISC. This is followed by rapid cleavage of typical caspase, caspase-3, or others, which, in turn, initiates the cleavage of various substrates in the cell. In type II signaling, little DISC is formed, and the caspase cascade is alternatively amplified by the mitochondria. The mitochondrion-mediated process involves activation of caspase-9 through the release of cytochrome *c* (9–12). Fas-mediated apoptosis of HSG cells was slightly inhibited by the caspase-9 inhibitor, although it was mainly inhibited by that for caspase-8 ($P < 0.05$ from *t*-test; Fig. 5, upper panel). These findings suggest that Fas-mediated apoptosis of HSG cells is partly mediated by the mitochondrion-mediated pathway, but mainly by the type I signaling pathway. However, in the case of the type I death signal caused by the caspase cascade initiated by the activation of caspase-8 at the DISC, the details of the DISC formation in HSG cells have not yet been clarified.

Apoptotic cell death of acinar and ductal epithelial cells is suggested to be one of the major mechanisms perpetuating loss of secretory function in patients with Sjögren syndrome (33–35). Therefore it is important to investigate the molecular interactions that regulate apoptosis of these cells, with the aim of developing specific immunotherapy for patients with this syndrome. Our results suggest that FasL or Fas, expressed on the cell surface of immune cells activated locally due to a disorder of the immune system, and cytokines such as TNF-α secreted from these cells, play an important role in inducing apoptosis in the salivary gland cells in these patients. Moreover, our results also suggest the potential of a new immunotherapy for certain kinds of oral cancers for which radiotherapy or anticancer drugs are currently being used, namely an immunotherapy utilizing cytokines and agonistic anti-Fas antibody or FasL-expressing activated immune cells, in combination.

In conclusion, the death of HSG cells induced by agonistic anti-Fas antibody CH-11 is through apoptosis, resulting from the activation of caspases followed by cleavage of PARP and ladder formation of DNA. The primary apoptosis signal is likely to be mainly mediated through caspase-8. The enhancement of the apoptosis by TNF-α pretreatment was attributed to the increased sensitivity of the cells to CH-11-stimulated apoptosis; this increase is caused by the induction of Fas protein by TNF-α and its recruitment to the cell surface.

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