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Biochemical and Biophysical Research Communications 353 (2007) 159–163
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The variation in Fas localization and the changes in Fas expression level upon stimulation with growth factors

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Received 23 November 2006
Available online 8 December 2006

Abstract

Although Fas (APO-1/CD95) is well known as a death receptor, its stimulation occasionally fails to induce apoptosis in malignant cells. On the contrary, Fas is reported to advance the cell cycle in cancer cells. Therefore, we investigated roles of Fas in cell growth and apoptosis using human lung cancer cell lines. Fas was localized in the cytoplasm in exponentially growing cells, whereas only confluent cells expressed Fas on the cell membrane. A stimulation of confluent cells by either of EGF, IGF-I or VEGF induced once a decrease in Fas expression level and its sequential recovery. Fas expression levels in confluent cells were negatively correlated with cell doubling times ($r = 0.757$, $p = 0.0088$). Fas remained on the cell membrane of IgM-treated cells even after the growth factor stimulation, leading to apoptosis with abnormal mitosis, whereas the same stimulation induced Fas internalization in IgG_1-treated cells. From these results, we suggest that Fas remaining on the cell membrane amplifies to induce apoptosis. Conversely, Fas internalization may enable cancer cells to escape from apoptosis. Our results suggest that growth factor may contribute to the resistance of cancer cells to Fas-mediated apoptosis in an autocrine or paracrine fashion.

Keywords: Fas; Growth factor; Apoptosis; Internalization

As Fas (APO-1/CD95), which is a 45-kDa type I membrane protein and belongs to the TNF receptor superfamily, has important role in apoptosis [1], it may be an attractive target for treating cancer cells. An activation of cytotoxic T lymphocytes expressing Fas [2] increases their Fas levels to induce apoptosis, resulting in their elimination [1]. The specific stimulation of Fas by anti-Fas antibodies (Abs) or Fas-ligand (Fas-L) induces an aggregation of intracellular death domains and then a recruitment of cytoplasmic adapter proteins, such as Fas-associated death domain (FADD/MORT1), finally leading to apoptosis [1,2]. The FADD contains both death and death effecter domains and is coupled on to Fas at the C-terminal of the death domain and procaspase-8 at the N-terminal of the death effecter domain. Scaffidi et al. have identified two cell types, each using almost exclusively one of two different Fas signaling pathways. In type I cells, caspase-8 was activated within seconds and caspase-3 within 30 min of receptor engagement, whereas in type II cells cleavage of both caspases was delayed for 60 min. However, both type I and type II cells showed similar kinetics of Fas-mediated apoptosis. But in type I cells it is initiated by large amounts of active caspase-8 formed at the death-inducing signaling complex (DISC) followed by direct cleavage of caspase-3. In contrast, in type II cells very little DISC and small amounts of active caspase-8 sufficient to induce the

Abbreviations: Ab, antibody; CLSM, confocal laser scanning microscope; EGF, epidermal growth factor; Fas-L, Fas-ligand; FCM, flow cytometry; Ig, immunoglobulin; IGF, insulin-like growth factor I; PBS, Ca^{2+}-, Mg^{2+}-free Dulbecco's phosphate-buffered saline; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

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doi:10.1016/j.bbrc.2006.11.145

apoptogenic activity of mitochondria are formed causing a profound activation of both caspase-8 and caspase-3. Only in type II cells can apoptosis be blocked by overexpressed Bcl-2 or Bcl-x_L [3,4]. However, details of this pathway are still unknown. As cancer cells express Fas *in vitro* and *in vivo* [5–8], cytotoxic T lymphocytes can putatively target Fas to eliminate cancer cells. Although proportions of apoptotic cancer cells are significantly correlated with Fas expression levels, cancer cells often secrete soluble Fas which can function as a Fas antagonist [9], and Fas is downregulated [10] or non-functional [11,12] in some cases. Furthermore, cancer cells are known to escape from the affection of cytotoxic T lymphocytes by secreting Fas-ligand (Fas-L) [11–13], and Fas has been reported to advance the cell cycle in cancer cells [14]. Thus, Fas has a controversial function in cancer cells. As it is important to know how Fas functions in cancer cells in order to map up cancer chemotherapy strategy, we focused on roles of Fas in cell growth and apoptosis in human lung cancer cell lines. In this study, we found that the cancer cell lines, together with growth factors, were closely correlated with cell growth by changing its cellular localization. Therefore, we discussed how we should evaluate exact situation of Fas together with growth factors to map up strategies of cancer treatment.

Materials and methods

Cell lines. The human lung cancer cell lines used were NPC-2, NPC-11, PC-10, and QG-56 squamous cell carcinomas, PC-3, NPC-4 and NPC-8 adenocarcinomas, and NPC-1, PC-6, and QG-90 small cell carcinomas [15] that were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS; Life Technologies, Grand Island, NY) (complete medium) in a humidified atmosphere of 5% CO_2 at 37 °C. The doubling times of these cell lines were measured by dye-exclusion method. Briefly, 5×10^4 cells in a 60 mm dish were incubated for 72 h. Then the cells were measured at least three times.

Analyses of protein levels. Cellular Fas levels were analyzed by measuring green fluorescence excitated by a 488-nm argon ion laser using a flow cytometer (Beckman Coulter, Miami, FL). After trypsinized, isolated confluent cells were fixed with ethanol and stained with anti-human Fas mouse monoclonal Ab (APO-1; DAKO, CA) and then with FITC-conjugated F(ab')2 fragment of rabbit anti-mouse Igs (Dako, Glostrup, Denmark) for 1 h each. Samples passed through 40-μm nylon mesh were subjected to flow cytometry.

Growth factor. The confluent cells were stimulated by 10 ng/ml EGF (Genzyme-Techne, Minneapolis, MN), 10 ng/ml (Upstate Biotech, Lake Placid, NY) or 5 ng/ml (Relia Tech GmbH, Braunschweig, Germany) for 5 min. Immediately after growth factor stimulation, medium was replaced to RPMI 1640 medium containing 3% FBS to prevent an influence of growth factors in FBS, and samples were subjected to further experiments.

Localizations of Fas and Golgi. Cells were fixed with 70% ethanol after preincubation in complete medium in 8-chamber slide (Nalge Nunc International, Naperville, IL) for 72 h to allow them to attach to the slide. First Abs used were anti-Fas mouse monoclonal Ab (DAKO), anti-Fas rabit polyclonal Ab [Fas (C-20); Santa Cruz], monoclonal anti-Golgi 58 k protein (Sigma–Aldrich, Saint Louis, Missouri), second Abs were fluorescein isothiocyanate (FITC)-conjugated anti-mouse Igs rabbit F(ab')2 (DAKO), R-phycoerythrin (RPE)-conjugated anti-mouse Igs swain F(ab')2, and FITC-conjugated anti-rabbit Igs swain F(ab')2 (DAKO). Cells were simultaneously stained with propidium iodide (PI), if necessary. Samples were subjected to confocal laser-scanning microscopy (Bio-Rad Laboratories, Hercules, CA).

Effects of Fas-specific Ab treatment. Effects of Fas-specific Ab on cell proliferation were analyzed by MTT assay. Anti-Fas IgM (clone, CH-11; MBL, Nagoya, Japan) or anti-Fas IgG_1 (clone, APO-1; DAKO) were used at 100, 300, 500 or 700 μg/ml for MTT. Confluent cells (originally 10^5 cells/ml) after 4-day preincubation were treated with anti-Fas Ab continuously for 24 h and then 10 μl of 5 mg/ml MTT [3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma) for 4 h followed by solubilizing the MTT-formazan product by adding 100 μl dimethyl sulfoxide (DMSO). Samples were measured using a microplate reader (ELx800; Bio-Tek, Denkendorf, Germany).

Results

Fas expression levels and growth factor influences

Cell doubling times (Fig. 1A) were correlated with Fas expression levels ($r = 0.757$, $p = 0.0088$) (Fig. 1B), indicating that cells expressing more Fas grow more slowly. Fas was localized diffusely in the cytoplasm of logarithmically growing cells (Fig. 1F, a) and on the cell membrane of confluent cells (Fig. 1F, b). As Fas expression levels were correlated with cell doubling times and Fas localizations differed between logarithmically growing and confluent cells, we examined effects of growth factors on Fas expression levels.

Confluent adenocarcinoma cells expressing more Fas on the cell membrane were stimulated by either of EGF, IGF, or VEGF. Fas decreased by EGF and VEGF in PC-3 cells, by VEGF in NPC-4 cells, and by all three growth factors in NPC-8 cells. Fas levels rapidly decreased once and were recovered 8 h thereafter (Fig. 1C–E). Thus, the growth factor stimulation was accompanied by Fas reduction, even though Fas was not directly stimulated.

To interpret why Fas levels decreased after growth factor stimulation, we observed Fas localization after the growth factor stimulation and found that Fas localized on the cell membrane was internalized and then disappeared once (Fig. 1F, c–e). Subsequently, Fas reappeared in the cytoplasm close to the nucleus 2 h thereafter (Fig. 1F, f–g). Simultaneous staining of Fas and Golgi apparatus with FITC (green) and RPE (red), respectively, showed that Fas was localized to the Golgi apparatus (Fig. 1F, h). The phenomenon was similarly observed in other cell lines (data not shown). Thus, the growth factor stimulation induced Fas internalization and sequential degradation independently of Fas stimulation.

Effects of anti-Fas Abs on cell growth and Fas expression

As Fas-induced apoptosis has been reported to depend on the kind of anti-Fas Ab used, effects of anti-Fas IgM and IgG_1 on cell growth were examined using adenocarcinoma cell lines expressing sufficient Fas. Although both Abs inhibited cell growth more prominently with increasing Ab concentrations, anti-Fas IgM inhibited cell growth more than Anti-Fas IgG_1 (Fig. 2A–C). To clarify why anti-

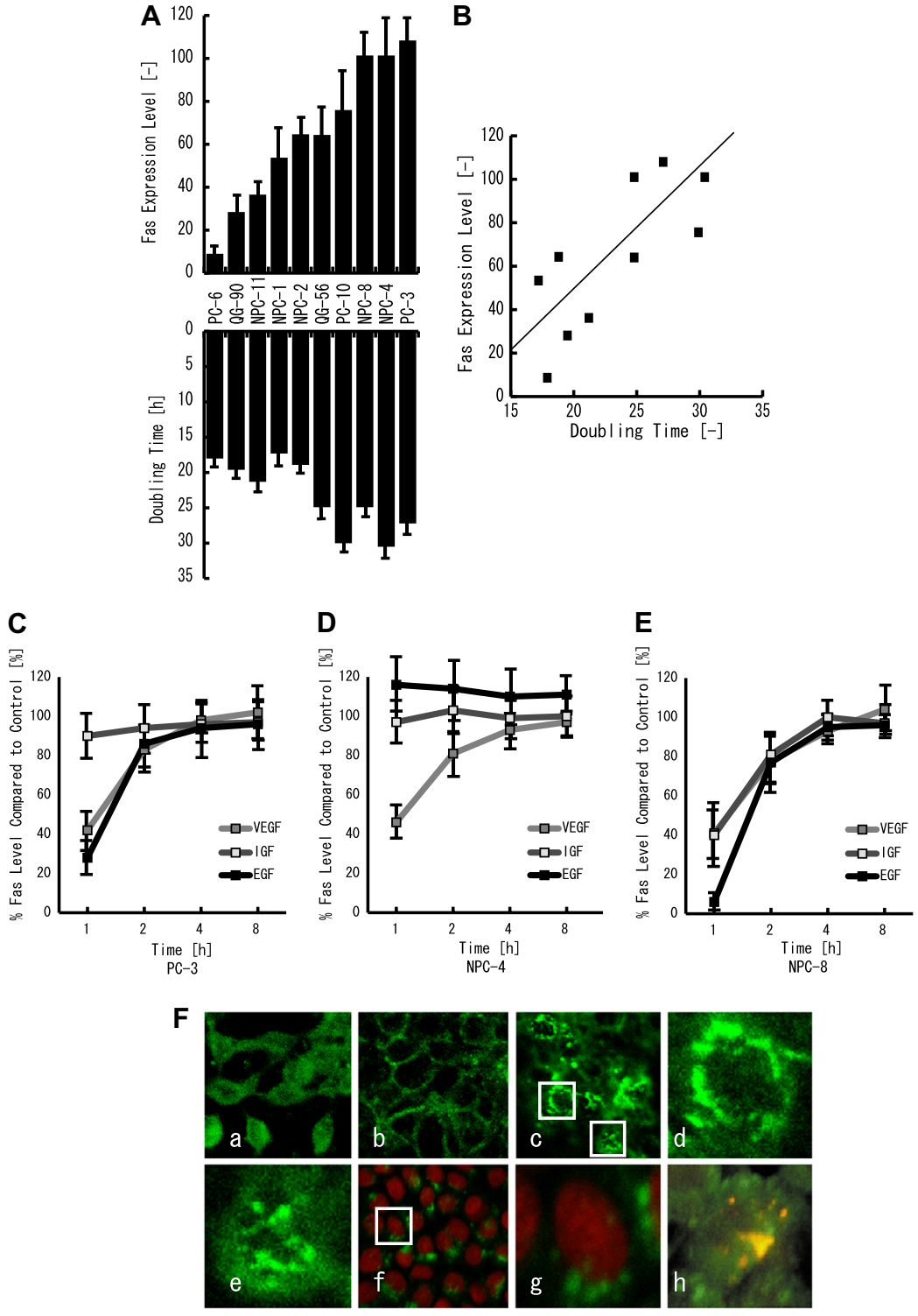

Fig. 1. Fas expression levels and growth factor influences. (A, B) Expression level of Fas in human lung cancer cell lines and its correlation with doubling time. (C–E) The influence of growth factor stimulation on Fas expression. Cells stimulated by growth were stained with anti-Fas Ab and measured by flow cytometry. (F) Changes in localization of Fas (FITC, green) according to the status of cell growth in NPC-2 squamous cell carcinoma cells. Cells were observed in logarithmic growth (a) and confluent cells (b). Cells were observed 1 h (c–e) or 2 h (f–h) after the stimulation. Cells were stained with anti-Fas Ab (green) and PI (red) (f–g). Cells were stained with anti-Fas Ab (red) and anti-Golgi Ab (green) (h). (For interpretation of color mentioned in this figure the reader is referred to the web version of this article.)

Fas IgM could more induce apoptosis than anti-Fas IgG, we serially observed localizations of anti-Fas IgM and anti-Fas IgG_1, i.e. the localization of Fas binding to anti-Fas IgG or IgM. Although IgG_1 was internalized after 1-h treatment, IgM remained on the cell membrane even after 1-h treatment and induced abnormal mitosis leading

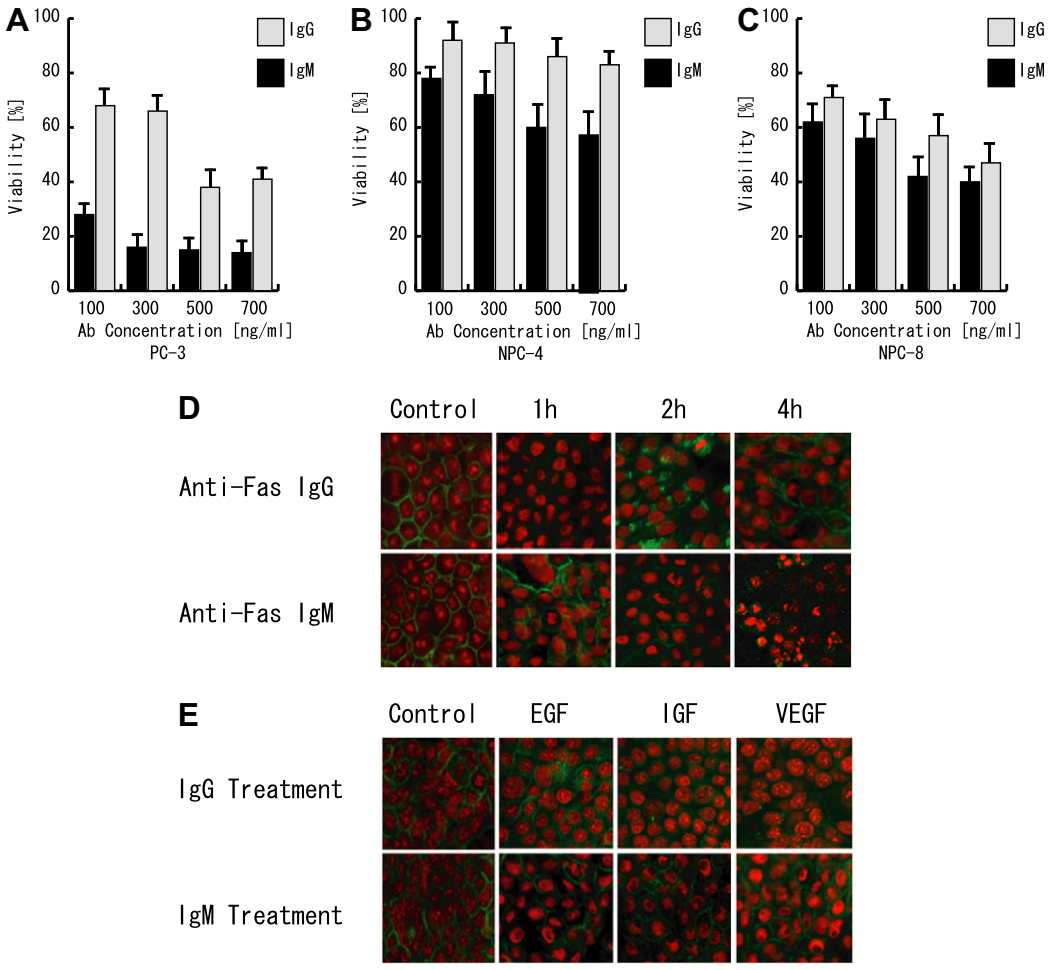

Fig. 2. Effects of anti-Fas Ab treatment. (A–C) Viability of cells treated with anti-Fas IgM or IgG_1 by MTT assay. (D) Localization of Fas after anti-Fas Ab treatment. Morphological changes in confluent cells treated with anti-Fas IgM (lower panels) or IgG_1 (upper panels) were stained with anti-Fas Ab (FITC, green) and PI (red). Typical apoptotic bodies can be seen in lower panels. (E) Changes in Fas localization in confluent cells previously stained with anti-Fas Ab for 1 h and then stimulated with each growth factor for 5 min. (For interpretation of color in this figure the reader is referred to the web version of this article.)

to apoptosis (Fig. 2D). As Fas was internalized after growth factor stimulation, we sequentially treated cells with anti-Fas IgM and a growth factor. Fas internalization was decreased by the treatment, and it remained on the cell membrane in anti-Fas IgM-treated cells (Fig. 2E). Thus, it seemed that anti-Fas IgM treatment allowed Fas to remain on the cell membrane to induce apoptosis, whereas Fas was internalized by treating with anti-Fas IgG, indicating that Fas to remain on the cell membrane long may be amplified to induce apoptosis. The examination was performed with NPC-8 cells, because the Fas was decreased by all three growth factors.

Discussion

Our findings in this study were as follows: (1) Fas levels in human lung cancer cell lines were associated with their doubling times. (2) Fas existed in the cytoplasm of logarithmically growing cells and on the cell membrane of confluent cells. (3) Growth factor stimulation induced Fas internalization. (4) Fas remained on the cell membrane of IgM-treated cells although after growth factor stimulation.

Fas may be an attractive target for cancer treatment as Spets et al. have reported that anti-Fas monoclonal Ab and its enhancement by IFN-γ induce Fas-mediated apoptosis [16]. However, as many kinds of normal cells express Fas *in vivo*, anti-Fas Ab treatment may possibly cause severe side effects [17–19]. Even if this obstacle could be removed, we found many problems to resolve because we found that the reduction of Fas levels was observed after Fas internalization induced by growth factor stimulation in human lung cancer cells. As this Fas internalization occurred immediately after the growth factor stimulation, it is likely that Fas internalization is accompanied with growth factor internalization. Therefore, our results partially compensate for the reports as described above.

As growth factor receptor is internalized through an endocytosis after binding of its ligand, it is possible for Fas to be involved in growth factor endocytosis. However, this phenomenon may occur not at random but inevitably

because Fas on the cell membrane was once completely internalized after growth factor stimulation. This is, at least, one of the reasons why growth factors inhibit Fas-mediated apoptosis [20,21]. Fas was also internalized by anti-Fas IgG Ab treatment. If this Fas internaliztion would be accompanied with growth factor receptor internalization was not determined, because our concern was focused on Fas localization and function. Anyway, many factors may affect Fas internalization. On the other hand, internalized Fas reappeared in the Golgi apparatus, indicating that Fas was newly synthesized. Thus, Fas may be recruited in the Golgi apparatus, stored in the cytoplasm during logarithmical cell growth, translocated onto the cell membrane, and internalized together with growth factor receptor stimulation or Fas stimulation.

It seemed that Fas to remain longer on the cell membrane amplified to fill its primary role as the death receptor. Fas was expressed on the cell membrane of confluent cells that were treated with anti-Fas IgG or IgM. Anti-Fas IgG treatment induced Fas internalization by growth factor stimulation, but anti-Fas IgM treatment allowed Fas to remain on the cell membrane longer and induce apoptosis although cells were stimulated by growth factor. We treated cells with 500 ng/ml anti-Fas IgG_1 and IgM containing approximately 2.0×10^9 and 3.4×10^8 molecules/cell, and these molecules were sufficient enough to saturate whole Fas. Our results are coincident with the report that there is a difference in capacity to induce apoptosis between anti-Fas IgG_1 and anti-Fas IgM recognizing same epitope [22].

From our results, we suggest that it is necessary to clarify the exact mechanisms of Fas remaining longer on the cell membrane and being associated with growth factor stimulation. As Fas localization may be closely related to its function, we should investigate exact mechanisms of Fas translocation in the next study.

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