

## Decrease of CD93 expression on the human monocyte-like cell line U937 treated with anti-Fas monoclonal antibody

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### Abstract

Human CD93, a receptor for complement component 1, subcomponent q (C1qRp), was shown to be selectively expressed by cells of a myeloid lineage, and was originally reported to be involved in the C1q-mediated enhancement of phagocytosis in innate and adaptive immune responses. The modulation of CD93 expression also has been investigated in various cells, in particular, in granulocytes and monocytes. However, its modulation in myeloid cells with apoptotic properties remains poorly understood. In this study, using flow cytometry and two types of CD93 monoclonal antibodies (mAbs), mNI-11 and X-2, we demonstrated the modulation of CD93 expression on the human monocyte-like cell line (U937) treated with an anti-Fas mAb, a substance for induction of apoptosis through the Fas/Fas ligand (FasL) system. The U937 cells expressed an apoptosis-related molecule, APO 2.7 after treatment with the anti-Fas mAb, and the expression of CD93 defined by mNI-11 or X-2 mAb on these cells was dramatically decreased. Furthermore, the decrease of CD93 expression on the U937 cells treated with anti-Fas mAb occurred in a time-dependent manner. Taken together, these findings suggest that the decrease of CD93 expression on the U937 cells treated with anti-Fas mAb would be a good model for analyzing immunological responses in myeloid cells with apoptotic properties under C1q and CD93 interactions.

Key words : anti-Fas monoclonal antibody, APO2.7, apoptosis, CD93, U937 cells

### Introduction

Human CD93 is a receptor for complement component 1, subcomponent q (C1qRp), and was originally reported to be involved in the C1q-mediated enhancement of phagocytosis<sup>1-4)</sup>. CD93 has a molecular weight (m.w.) of about 90- 100 kDa, and is a heavily O-glycosylated type I transmembrane protein consisting of unique C-type lectin domains<sup>5)</sup>. CD93 has been shown to be selectively expressed on myeloid cells, such as granulocytes, monocytes and endothelial cells, suggesting that this molecule may be involved in some important biological functions in several immune responses<sup>6-8)</sup>.

The modulation of CD93 expression has been

investigated in a variety of cells, particularly in granulocytes and monocytes, and rapid up-regulation of the expression of this molecule by the inflammatory peptide FMLP has been reported<sup>9)</sup>. We also reported that CD93 on the human monocyte-like cell line (U937) was strongly up-regulated following exposure to the protein kinase C (PKC) activator, phorbol myristate acetate (PMA), and this regulation was controlled by the PKC delta isoenzyme<sup>10)</sup>.

On the other hand, apoptotic cells induced by some stimulants are continuously occurring in the living organism, and in order to maintain homeostasis these cells are scavenged by phagocytic cells, such as granulocytes, monocytes and macrophages. As CD93 on

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myeloid cells is involved in C1q-mediated phagocytosis, these cells with apoptotic properties cannot clear various foreign antigens under C1q and CD93 interactions. In addition, it has also been reported that scavenging of apoptotic cells failed to occur in CD93-knock out mice<sup>11)</sup>. Following these findings, analysis of the modulation of CD93 expression on the myeloid cells with apoptotic properties should deliver some important information about the scavenging activity in several immune responses. However, modulation of the CD93 expression on myeloid cells with apoptotic properties currently remains unknown.

Fas (APO-1: CD95) is a 45 kDa type I membrane protein, and Fas ligand (FasL) is a 37-40 kDa type II membrane protein, both of which belong to the tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) receptor and ligand families<sup>12, 13)</sup>. Activation of Fas mediated by FasL, such as anti-Fas antibodies, induces apoptosis of various Fas-bearing cells through activation of the caspase cascade<sup>14)</sup>. This Fas/FasL system interaction constitutes one of the main systems regulating immune responses and homeostasis<sup>15)</sup>.

In this study, using flow cytometry, we investigated the modulation of CD93 on U937 cells treated with an anti-Fas monoclonal antibody (mAb) accompanied by apoptotic properties using two types of CD93 mAbs, mNI-11<sup>16)</sup> and X-2<sup>17)</sup> to define the roles of the CD93 on apoptotic cells in various immune responses.

## Materials and Methods

### Cell line

The human monocyte-like cell line (U937) used in the present study was supplied by the Health Science Research Resource Bank (HSRRB) (Tokyo, Japan). U937 cells were cultured in RPMI 1640 medium (GIBCO) supplemented with 10 mM Hepes buffer, 2 mM glutamine and 10% fetal calf serum (FCS) (referred to as complete medium).

### Reagents

A CD93 monoclonal antibody (mAb), mNI-11 (mouse IgG1)<sup>16)</sup>, an anti-Fas mAb, CH-11 (mouse IgM)<sup>18)</sup>, a fluorescence isothiocyanate (FITC)-conjugated goat anti-

mouse IgG antibody, isotype-matched normal mouse IgG1, and isotype-matched normal mouse IgM were purchased from MBL Co. (Nagoya Japan). A phycoerythrin (PE)-conjugated APO2.7 (mouse IgG1) mAb<sup>19)</sup> was purchased from Beckman Coulter (U.S.A). A CD93 mAb, X-2 (mouse IgG1)<sup>17)</sup> and a PE-conjugated isotype-matched normal mouse IgG1 were purchased from Cosmo Bio Co. (Tokyo, Japan).

### Treatment with the anti-Fas monoclonal antibody

The U937 cells ( $4 \times 10^6$ ) in complete medium were treated with an anti-Fas monoclonal antibody (mAb) (250 ng/ml), an apoptosis-inducing reagent, for 0, 3, 6, 8, 10, and 24 hrs in a CO<sub>2</sub> incubator. The cells were harvested at various times, and the modulation of CD93 defined by mNI-11 or X-2 was analyzed with flow cytometry. Apoptotic monitoring of the cells treated with the anti-Fas mAb (250 ng/ml) or isotype-matched normal mouse IgM (250 ng/ml) was performed by staining with an apoptosis-related mAb (APO2.7) under flow cytometry, as previously described<sup>17)</sup>. The APO2.7 mAb can be used for the direct quantitation of apoptotic cells by flow cytometry.

### Flow cytometry

The U937 cells treated with the anti-Fas mAb or isotype-matched normal mouse IgM for 24 hrs were washed in cold phosphate-buffered saline (PBS) containing 0.1% NaN<sub>3</sub> (referred to as the washing buffer), and incubated in PBS containing 25% normal goat serum, 1 mg/ml normal human IgG and 0.1% NaN<sub>3</sub> to block the Fc receptor (FcR) for IgG for 10 min on ice.

After washing three times, the cells were incubated with the optimal concentration of PE-conjugated APO2.7 mAb for 30 min on ice. Negative controls (NCs) were provided by staining with PE-conjugated isotype-matched normal mouse IgG1. Thereafter, the U937 cells treated with the anti-Fas mAb or isotype-matched normal mouse IgM for 0, 1.5, 3, 6, 10, and 18 hrs were harvested and washed in washing buffer three times, after which 0.25% paraformaldehyde-PBS was added and incubated for 2 min on ice. After washing in the washing buffer, cells were incubated in PBS containing

25% normal goat serum, 1 mg/ml normal human IgG and 0.1% NaN<sub>3</sub> to block the FcR for IgG for 10 min on ice. The cells were washed in the washing buffer, and incubated with PE-conjugated APO2.7 mAb and digitonin 100 µg/ml for 30 min on ice. The cells were washed in the washing buffer, and gated for exclusion of cell debris and aggregates. The percentage of positively stained cells for APO2.7 mAb was determined by the use of a fluorescence-activated cell sorter (FACScan; Becton Dickinson). The experiment was repeated three times.

Modulation of CD93 expression on the U937 cells treated with anti-Fas mAb was analyzed using two types of mAbs (mNI-11 and X-2). The cells treated with anti-Fas mAb for 0, 3, 6, 8, 10, and 24 hrs were harvested at each of these times, and washed in the washing buffer. Thereafter, the cells were resuspended in PBS containing 25% normal goat serum, 1 mg/ml normal human IgG and 0.1% NaN<sub>3</sub> to block the FcR for IgG, and then the cells were incubated with an optimal concentration of mNI-11 or X-2 for 30 min at room temperature. Isotype-matched normal mouse IgG1 was used in each stage as a negative control. After washing twice in the washing buffer, the cells were incubated with an FITC-conjugated goat anti-mouse IgG antibody for 15 min at room temperature. Following a final wash with the washing buffer and resuspension in PBS containing 2% FCS and 0.1% NaN<sub>3</sub>, the cells were gated for exclusion of cell debris and aggregates. The percentage of positively stained cells for mNI-11 or X-2 was determined by the use of the FACScan system.

## Results and Discussion

In this study, we investigated the modulation of CD93 expression on U937 cells treated with anti-Fas mAb, an apoptosis-inducing substance, using two types of CD93 mAbs (mNI-11 and X-2) and flow cytometry. Fas, a type I membrane protein belonging to a member of the TNF- $\alpha$  receptor superfamily<sup>12, 13)</sup>, is expressed by macrophages, dendritic cells (DC), fibroblasts, lymphocytes, and also U937 cells. Activation of Fas mediated by the Fas ligand (FasL), such as anti-Fas mAb, induces apoptosis of Fas-bearing cells through several caspase cascades<sup>14)</sup>. First of all, the presence of

an apoptosis-related molecule (APO2.7) on U937 cells 24 hrs after treatment with the anti-Fas mAb (250 ng/ml) was monitored without permeabilization by digitonin using a PE-conjugated APO2.7 mAb and flow cytometry. As shown in Fig.1, the U937 cells treated with the anti-Fas mAb strongly expressed the APO2.7 molecule (approximately 60%), indicating that some populations among the U937 cells treated with the anti-Fas mAb shifted to an apoptotic cell stage. In addition, the APO2.7 molecule on the U937 cells treated with anti-Fas mAb (250 ng/ml) for various times (0, 1.5, 3, 6, 10, and 18 hrs) was monitored with permeabilization by digitonin using a PE-conjugated APO2.7 mAb and flow cytometry. Fig.2 shows that the U937 cells treated with the anti-Fas mAb clearly expressed the APO2.7 molecule at 3 hrs after anti-Fas mAb treatment. It has been reported that APO2.7 mAb is recognized in the mitochondrial membrane at an m.w. of 38 kD, and is useful as a marker for the detection of apoptotic cells<sup>19)</sup>. Furthermore, the APO2.7 mAb can be used for direct quantitation of apoptotic cells by flow cytometry, with or without permeabilization by digitonin. The use of the APO2.7 molecule allows for precise monitoring of the early and late cellular responses during apoptosis<sup>19-21)</sup>.

Next, we demonstrated the modulation of CD93 expression on U937 cells treated with anti-Fas mAb using two types of CD93 mAbs (mNI-11 and X-2). Figure 3 shows that the CD93 molecule defined by mNI-11 or X-2 mAb on these cells dramatically decreased at 24 hrs. CD93 has an m.w. of 90- 100 kDa, and is a heavily O-glycosylated type I transmembrane protein consisting of unique C-type lectin domains<sup>5)</sup>. In addition, the O-glycosylation of the CD93 molecule is a very important factor for the stability of this molecular expression<sup>22)</sup>. From these findings, we suggest that the CD93 molecule glycosylation sites may be affected by apoptosis signal(s) through the Fas/FasL system, and as a result, CD93 expression on U937 cells treated with anti-Fas mAb significantly decreased. Moreover, as shown in Fig. 4, we provided the evidence that the CD93 expression on the U937 cells treated with anti-Fas mAb decreased in a time-dependent manner, suggesting that CD93 expression on these cells strongly decreased in the late phase after anti-Fas mAb treatment. Recently, we reported that the expression of CD93 on the

granulocytes of patients with liver cirrhosis, chronic hepatitis B or C and hepatocellular carcinoma significantly decreased as compared with that in normal healthy donors <sup>23)</sup>. These findings suggest that the granulocytes of patients with liver diseases may be affected by the Fas/FasL system during the chronic inflammatory process.

The reason for the decrease of CD93 expression on the U937 cells treated with anti-Fas mAb remains unclear at the present time. However, we can suggest two possible reasons for this finding. One possible reason is signal transduction mediated by Fas. Fas recruits Fas-associated death domain (FADD) and caspase-8, which form the death-inducing signal complex that promotes apoptosis <sup>14)</sup>. Although Fas is traditionally viewed as a death receptor that triggers apoptosis, more recent studies have shown that, under certain circumstances, Fas can also activate the extracellular signal-regulated kinase (ERK) and NF- $\kappa$ B signal pathways <sup>24)</sup>. With this ability, Fas-related signal(s) can stimulate cell growth, differentiation and modulation of some cell surface antigens, including CD93, in various cell types.

Another possible reason for the decrease of CD93 expression is shedding of this molecule from the cell membrane after anti-Fas mAb treatment. Bearing in mind, as already stated above, that O-glycosylation is important for the stability of CD93 expression <sup>22)</sup>, the decrease of CD93 expression on the U937 cells treated with anti-Fas mAb could well depend on unglycosylation. Recently, it was reported that CD93 expressed on monocytes and neutrophils is susceptible to the PKC activator, PMA <sup>25)</sup>. In addition, this report indicated that PMA-induced CD93 shedding on monocytes was accompanied by a decrease in CD93 cell surface expression. Thus, decrease of CD93 expression on the U937 cells treated with anti-Fas mAb may be affected by phosphorylation of some proteins by PKC action. As a result of phosphorylation by some protein kinases, CD93 expression on the U937 cells treated with anti-Fas mAb might be strongly reduced. More recently, we also provided proof that CD93 expression on the U937 cells was down-regulated by a PKC delta isoenzyme using a PKC delta inhibitor, Rottlerin <sup>10)</sup>.

The molecular and biological functions of CD93 have

been investigated by knock-out mice lacking CD93 expression <sup>11)</sup>. The findings from this study suggest that CD93-deficient mice lacked the ability to remove impaired apoptotic cells *in vivo*. As CD93 is involved in the C1q-mediated phagocytosis, analysis of the modulation of CD93 expression on the U937 cells with apoptotic properties induced by the Fas/FasL system will be a good model for the clearance of various foreign antigens under the C1q and CD93 interactions.

In conclusion, we found a dramatic decrease in CD93 expression on the U937 cells treated with anti-Fas mAb through the Fas/FasL system. Further analyses are needed to demonstrate the detailed intracellular mechanism(s) of this finding at the molecular and cellular levels.

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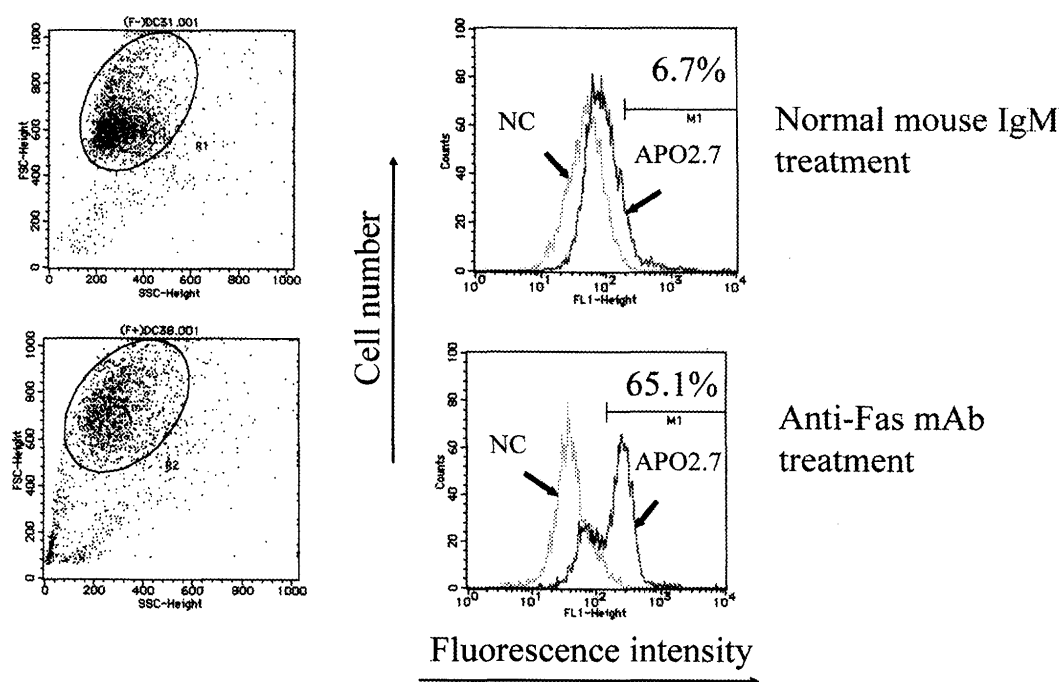


Figure 1. Expression of apoptosis-related APO2.7 molecule in the U937 cells treated with anti-Fas mAb. The U937 cells were treated with an anti-Fas mAb (250 ng/ml) or isotype-matched normal mouse IgM (250 ng/ml) for 24 hrs. The cells were harvested, and stained with PE-conjugated APO2.7 mAb without permeabilization by digitonin. Negative controls (NCs) were provided by staining with PE-conjugated isotype-matched normal mouse IgG1. The cells were gated for exclusion of cell debris and aggregates, and the percentage of positively stained cells for the APO2.7 mAb was determined using FACScan.

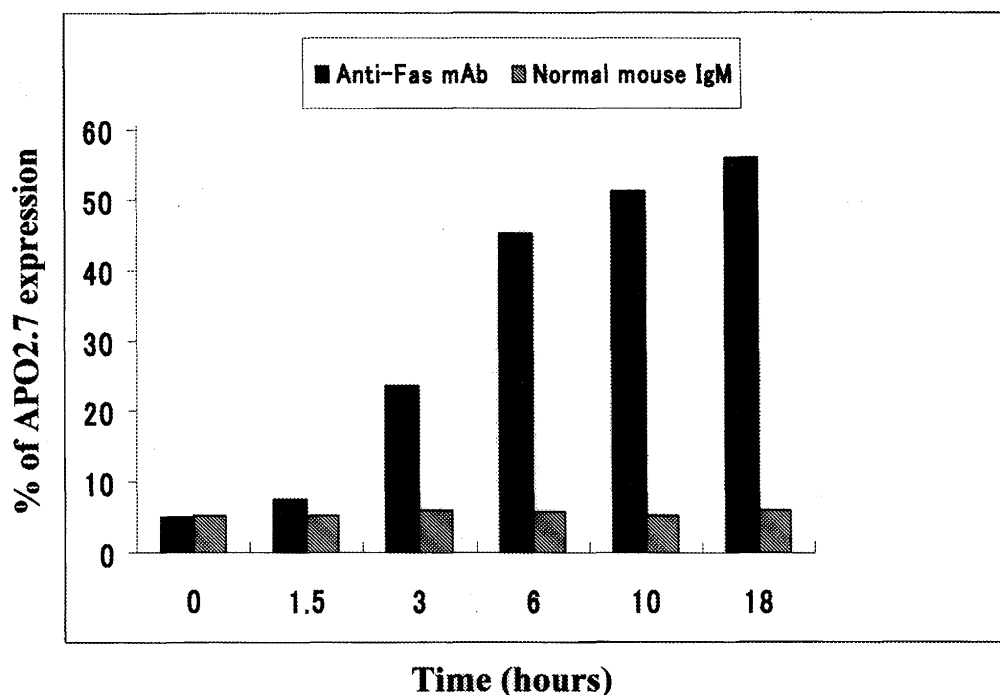


Figure 2. Expression of apoptosis-related APO2.7 molecule in the U937 cells treated with anti-Fas mAb occurs in a time-dependent manner. The U937 cells were treated with an anti-Fas mAb (250 ng/ml) or isotype-matched normal mouse IgM (250 ng/ml) for 0, 1.5, 3, 6, 10, and 18 hrs. The cells were harvested, and stained with PE-conjugated APO2.7 mAb with permeabilization by digitonin. The cells were gated for exclusion of cell debris and aggregates, and the percentage of positively stained cells for the APO2.7 mAb was determined using FACScan.

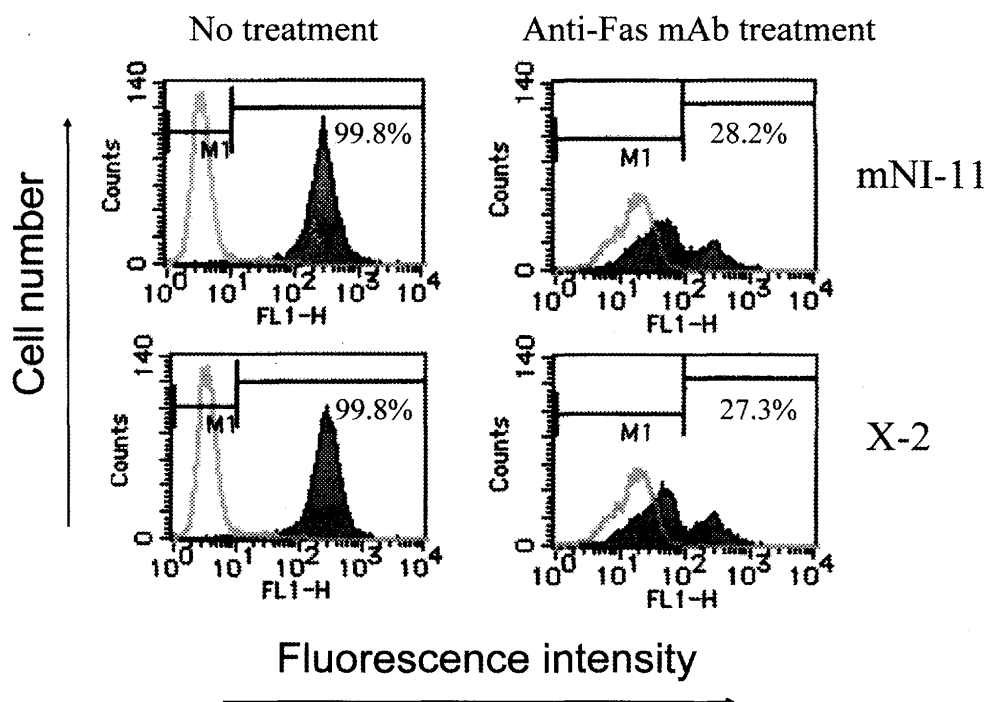


Figure 3. Decrease of CD93 expression on the U937 cells treated with anti-Fas mAb. The U937 cells were treated with anti-Fas mAb (250 ng/ml) for 24 hrs. The cells were harvested, and stained with CD93 mAbs (mNI-11 and X-2) and an FITC-conjugated goat anti-mouse IgG (shaded histograms). Negative controls (NCs) (opened histograms) were provided by staining with isotype-matched normal mouse IgG1. The cells were gated for exclusion of cell debris and aggregates, and the percentage of positively stained cells for the CD93 mAbs (mNI-11 and X-2) was determined using FACScan.

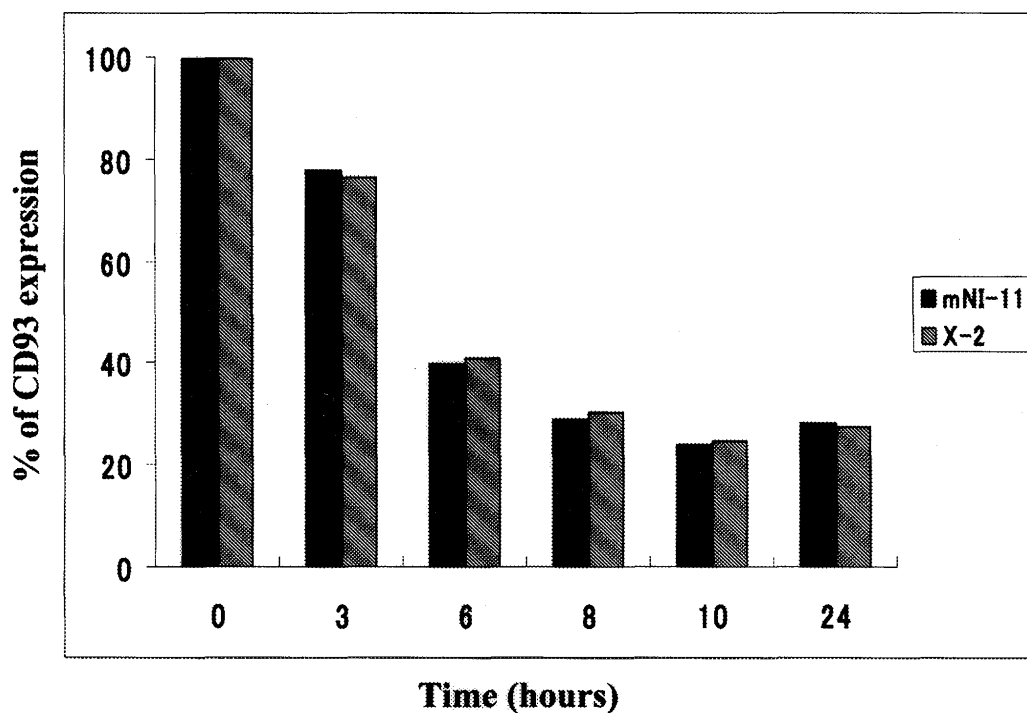


Figure 4. Decrease of CD93 expression on the U937 cells treated with anti-Fas mAb occurs in a time-dependent manner. The U937 cells were treated with anti-Fas mAb (250 ng/ml) for various times. The cells were harvested at 0, 3, 6, 8, 10, and 24 hrs, and stained with CD93 mAbs (mNI-11 and X-2) and an FITC-conjugated goat anti-mouse IgG. Negative controls (NCs) were provided by staining with isotype-matched normal mouse IgG1. The cells were gated for exclusion of cell debris and aggregates, and the percentage of positively stained cells for the CD93 mAbs (mNI-11 and X-2) was determined using FACScan.



## 抗Fasモノクローナル抗体処理単球系細胞株(U937)表面上のCD93分子の発現低下

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### 要旨

抗Fasモノクローナル抗体処理単球系細胞株(U937)表面上の補体(C1q)レセプター(CD93)の発現を我々が開発したCD93モノクローナル抗体(医学生物学研究所で販売)とフローサイトメトリー法を用いて解析した。その結果、抗Fasモノクローナル抗体処理U937細胞はアポトーシスマーカーであるAPO2.7分子の発現を誘導すると共に、細胞表面上のCD93分子の発現を著明に低下させた。また、この発現低下は抗Fasモノクローナル抗体の処理時間に依存していた。以上の結果から、抗Fasモノクローナル抗体処理単球系細胞株(U937)はFas/Fas ligand (Fas/Fas L)システムを介したCD93分子の発現調節を考察する上で非常に有益な情報を提供するものと考えられる。

キーワード：抗Fasモノクローナル抗体、APO2.7、アポトーシス、CD93、U937細胞