# Cell cycle specificity of Fas-mediated apoptosis in WIL-2 cells

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**Abstract Antibodies to Fas/APO1 receptor induce effective apoptosis in WIL-2 cells of the human B-lymphoid line. Quantitative assessment of the extent of the death in cells synchronized by thymidine block revealed a significant increase in their sensitivity to the cytocidal effect mediated by Fas/APO1 during the $G_1$ phase of the cell cycle. Western analysis of the content of the p53 antigen in the cytoplasm and nuclei of the cells showed that the Fas/APO1-induced death is accompanied by massive translocation of the p53 from the cytoplasm to the nucleus. These findings suggest that cell vulnerability to the Fas/APO1-mediated apoptosis is subjected to regulation by cell cycle-dependent mechanisms, one of which is probably the function of the p53 antigen.**



*Key words:* Fas/APO1-mediated apoptosis; Cell cycle; p53 antigen

## 1. Introduction

Apoptosis of dividing cells has been shown to occur at various phases of the cell cycle depending on cell type and/or the nature of a stimulus [1–5]. In general, any of inducers results in the disturbance of gene expression or their product function and it is considered to be a reason of an imbalance between the growth-inhibitory and the growth-promoting signals [1,6–8]. However, the mechanisms of such an interrelation of cell death and proliferation are still obscure.

The Fas/Apo-1/CD95 cell surface receptor belongs to the nerve growth factor/tumor necrosis factor (TNF) receptor family and can signal apoptosis [9–11]. Evidence has been presented recently for a crucial role of Fas antigen and its ligand in natural killer-mediated cytotoxicity, T-lymphocyte selection and T-cell receptor-induced apoptosis [12–16]. Fas has been shown to trigger death in a variety of non-lymphoid cell lines upon specific antibody (anti-Fas Abs) binding [3,17–19]. In these cases usually the time of realisation of Fas-mediated apoptosis (12–20 h) is comparable to the cell doubling time period, which is consistent with the possibility of an involvement of cell cycle regulatory mechanism(s) in death.

This study was undertaken to investigate the possible cell-cycle specificity of Fas-mediated signalling. The results indicate that cells in the $G_1$ stage are more sensitive to anti-Fas Abs-induced death. Such a vulnerability is perhaps related to the p53-dependent activation of death genes.

## 2. Materials and methods

$G_1$/S blockade of WIL-2 cells was achieved by incubation of cells in RPMI-1640 medium supplemented with 10% dialysed fetal calf serum, 100 units/ml penicillin, 100 mg/ml streptomycin and 5 mM thymidine for 16 h. Then the cells were washed twice and the medium was replaced by RPMI-1640 with 10% FBS and antibiotics. The anti-Fas IgM (Upstate Biotechnology, USA) were added at various time points after thymidine removal. Aliquots of the cells for cell cycle analysis and cell viability determination were taken every 3 h. To arrest cells in the $G_1$ phase mimosine was added to the medium at a final concentration of 75 mM for 10 h [20]. After washing, at various time periods the WIL-2 cells were rinsed and their cell cycle distribution and sensitivity to the cytotoxic effect of anti-Fas antibodies were determined. FACS analysis of propidium iodide-stained cells was used for assessment of WIL-2 cell viability [21]. The percentage of dead cells was calculated as follows:

$$\frac{(C_t - E)}{C_t} \times 100$$

where $C$ is the number of viable cells in the control sample and $E$ in the sample treated with anti-Fas IgM; $t$ is time of treatment.

Cell cycle was analysed as described [22].

The cytoplasmic and nuclear fractions from WIL-2 cells were prepared by hypotonic shock as follows [23]. Control and anti-Fas Abs-treated cells were lysed in 10 mM Tris-HCl, pH 7.5, 0.5% Nonidet P-40, 10 mM NaCl, 1.5 mM MgCl, 1 mM PMSF and pelleted by centrifugation at $30\,000 \times g$ for 30 min. The supernatant was collected and used as a cytoplasmic fraction. The nuclear fraction was extracted from the pellet by 10 mM Tris-HCl, pH 7.5, 1.5 mM MgCl and 0.5 M NaCl at 4°C for 20 min. The cytoplasmic and nuclear fractions were normalized for protein concentration by the method of Bradford [24] and analysed by Western blotting with anti-cyclin D1, anti-c-*myc* (Santa Cruz Biotech.) and anti-p53 (a gift of Dr. P. Chumakov, Institute of Molecular Biology, RAS, Moscow) antibodies followed by probing with horseradish peroxidase-conjugated goat anti-mouse antibodies.

## 3. Results and discussion

WIL-2 cells are the suspension B lymphoblastoid cell line and can be induced by anti-Fas Abs to apoptosis without any additional sensitizing treatment. Although the dying cells reveal the typical morphological features of apoptosis, no internucleosomal DNA degradation is observed (data not shown). The absence of the soluble DNA fragments makes it easy to analyse the cell cycle phase of dying cells without taking special precautions to prevent the loss of DNA contents. We therefore selected this particular cell line for assessing the relationship between the cell cycle stage and the sensitivity of cells to anti-Fas Abs.

Initial attempts to synchronize the WIL-2 cells by isoleucine deprivation or using hydroxyurea or aminopterine as blocking agents were unsuccessful because of their high cytotoxicity. However, by thymidine arrest we did manage to reach effective synchronization manifested in synchronous entry of up to 80% of cells to the S phase immediately after nucleoside removal (Fig. 1). To make the following figures more readable we marked cells 0, 1.5, 3, 6 and 9 h after a cancel of blockade as $G_1$/S, S, S/$G_2$, M/$G_1$ and $G_1$ populations, respectively.

Testing the effect of anti-Fas antibodies on WIL-2 cells that

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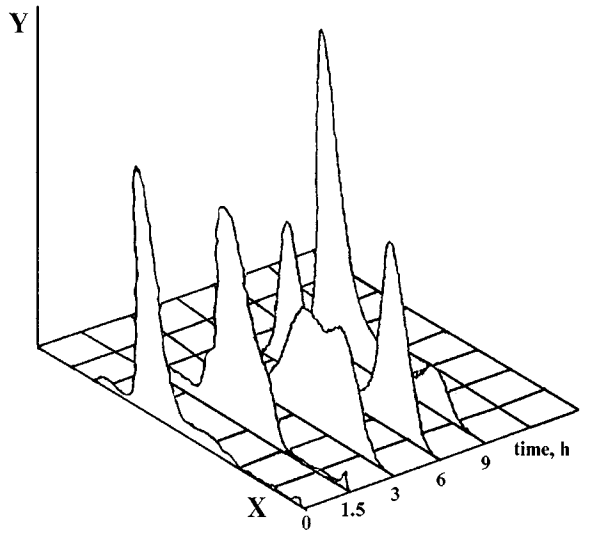


Fig. 1. Cell cycle distribution 0, 1.5, 3, 6 and 9 h after blockade removal. *X*-axis, DNA content; *Y*-axis, cell number.

were arrested at the $G_1$/S border with 5 mM thymidine at various time points after thymidine removal revealed a significant change in their sensitivity throughout the cell cycle. It turned out that the extent of the specific antibody cytotoxicity was increased in the order $G_1/S < G_2 < M/G_1 <$ 'second' $G_1$ (Fig. 2). A similar picture, though less prominent, was observed when anti-Fas Abs were applied at the lower concentration (data not shown). There are at least two possible explanations of the effects revealed. First, cells should pass the phase of mitosis and die afterwards; and second, cells in $G_1$ are more sensitive to the cytotoxic effect of anti-Fas antibodies.

To test these suggestions the experiments were carried out with another blocking agent, mimosine. It is a rare amino acid that can arrest mammalian cells in $G_1$ [20]. After the mimosine removal cells completed the $G_1$ stage of the cell cycle and entered into the S phase within 3 h. They kept the synchronous movement throughout the cell cycle up to 12 h after the treatment, and desynchronized after mitosis (data not shown). Fig. 3 indicates that anti-Fas antibodies added to cells in early

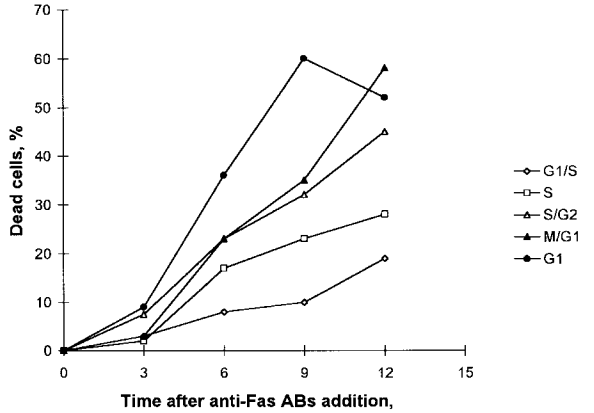


Fig. 2. Kinetics of Fas-mediated death of WIL-2 cells at various stages of the cell cycle. Anti-Fas Abs were added to cells 0, 1.5, 3, 6 and 9 h after thymidine removal, and viability was measured every 3 h (see Section 2). The values represent data of at least two independent experiments.

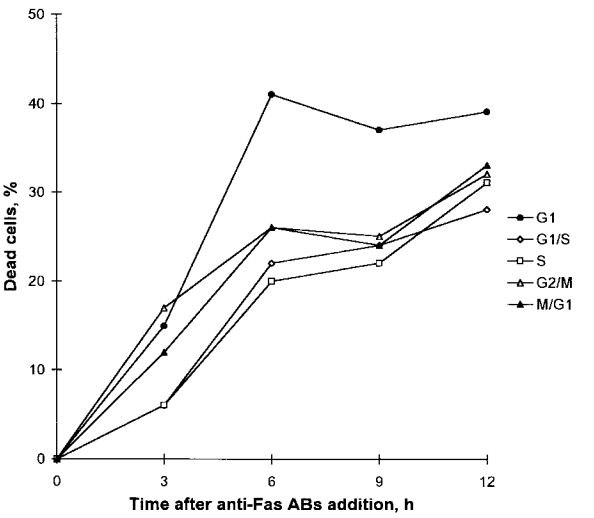


Fig. 3. Kinetics of Fas-mediated death of WIL-2 cells at various stages of the cell cycle. Anti-Fas Abs were added to cells 0, 3, 6, 9 and 12 h after mimosine removal, and viability was measured every 3 h (see Section 2). The values represent data of at least two independent experiments.

middle $G_1$ induced a more prominent cytotoxicity than in other phases of the cell cycle. Thus, the experiments with two different blocking agents show that the sensitivity of WIL-2 cells increased the closer they were to some point in $G_1$.

The highest cytotoxicity in $G_1$ is obviously determined by an involvement of cell cycle-depending protein(s) in Fas-mediated signal transduction. The Western analysis of possible candidates for the role of $G_1$-specific regulatory proteins, in particular cyclin D1, p53 and c-*myc* proteins, did not indicate the alterations of their expression in the synchronized cells (data not shown). However, taking into account the possible $G_1$-specific redistribution of p53 [25,26], the intracellular location of this protein was analysed. As one can see in Fig. 4, the bulk of p53 was revealed in the cytoplasmic fraction of control WIL-2 cells. The treatment with anti-Fas Abs resulted in the translocation of the protein into the nuclear fraction. One of the possible explanations is based on the assumption that p53 located in nuclei in $G_1$ activates the transcription of genes involved in Fas-mediated cytotoxic signalling. Thus, the preferential cell sensitivity in $G_1$ to anti-Fas Abs might be determined by the p53-dependent activation of 'cell death' genes. It should be noted that this explanation is valid only for the situation when anti-Fas-dependent cytotoxicity is observed in the absence of inhibitors of RNA and protein synthesis. At the same time, it cannot be excluded that p53 trans-

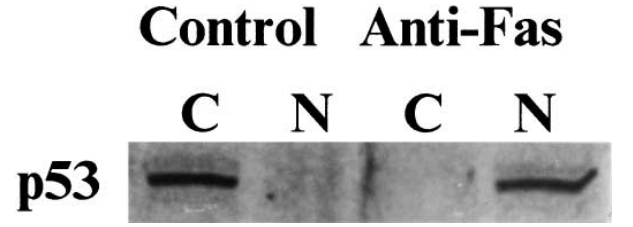


Fig. 4. p53 redistribution in cells treated with anti-Fas Abs. C, cytoplasmic fraction; N, nuclear fraction. Cells were extracted and subjected to Western blotting as described in Section 2.

location in dying WIL-2 cells is initiated by the appearance of the DNA damage [27]. Alternatively, it should be considered as a cell cycle-independent consequence of an apoptotic pathway realization. At present it is hard to say what step(s) in the Fas-mediated signal pathway is (are) cell cycle dependent. Besides the p53 translocation and the following gene activation, the function of the proteins discovered recently should be also considered [28,29,31]. Taking into account the significance of kinase and phosphatase activities in the mechanism of cell cycle regulation [30], it is attractive to speculate on the phosphatase and kinase associated to Fas receptor [29,31] as a key factor linking the cell cycle mechanism and Fas-mediated apoptotic signalling.

As a conclusion it should be noted that the results obtained can be extended to further study of the possible therapeutic potential of the combined treatment of Fas-bearing tumor cells with anti-Fas antibodies and cell cycle blocking agents.

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