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Regulation of *Fas* gene expression in HeLa cells as determined by modified RT-PCR

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Abstract. We determined human *Fas* messenger RNA (mRNA) levels in HeLa cells using a 'mutagenic' reverse transcription–polymerase chain reaction, which quantitates mRNA levels using the corresponding genomic DNA as an internal control. The expression level of Fas mRNA was very low in serum-deprived quiescent HeLa cells. In conjunction with the start of cell-cycle progression upon the addition of serum to culture medium, the *Fas* mRNA level gradually increased, reached its peak at 36 h and returned to the basal level after 48 h. HeLa cells at 36 h exhibiting a high level of *Fas* mRNA expression were more susceptible to the anti-Fas antibody apoptotic signal. Thus, the regulation of Fas expression is associated with cell-cycle progression, and this method for *Fas* mRNA detection may be useful, particularly for the analysis of small amounts of samples.

Key words. Apoptosis; cell cycle; mRNA; anti-Fas antibody; quantitative assay.

Fas is a member of the tumour necrosis factor receptor family [1] and is expressed on activated lymphocytes as well as in several tissues. When either Fas ligand binds to or anti-Fas antibody cross-links with Fas, signal transduction which eventually leads to apoptosis is induced [2]. Apoptosis is physiological cell death which regulates organ development and homeostasis. The Fas–FasL system is involved in clonal deletion and/or the activation-induced suicide of mature T cells [3, 4], and also functions as a regulator of apoptosis in liver [5]. A sensitive method for the detection of Fas gene expression would therefore be quite useful for understanding the mechanism of apoptosis. Northern blotting is generally used for the detection of *Fas* mRNA [6], but detecting low-level expression by Northern blotting in small amounts of samples is generally difficult. Use of reverse transcription (RT)–polymerase chain reaction (PCR) has enabled the detection of mRNA from small samples. However, the use of this method for the quantitative analysis of mRNA is relatively limited because of the exponential nature of the amplification of the targeted nucleic acids. 'Competitive PCR' [7] or the 'PCR-aided transcript titration assay (PATTY)' [8] has been successfully used for the quantitation of mRNA in small samples. However, these methods are rather complicated and expensive to carry out. In the present study, we employed a 'mutagenic' RT-PCR [9] for the quantitative detection of human *Fas* mRNA. This method utilizes genomic DNA as an internal control by introducing a point mutation into the cDNA at the RT step. Using this system, we show that the expression of *Fas* mRNA in HeLa cells is regulated in association with cell-cycle progression.

Materials and methods

Cell culture and flow cytometry. HeLa cells were obtained from the American Type Culture Collection (Rockville, MD, USA), and grown in Dulbecco's modified Eagle's medium (DMEM) containing fetal calf serum (FCS). For flow cytometric analysis, cells were

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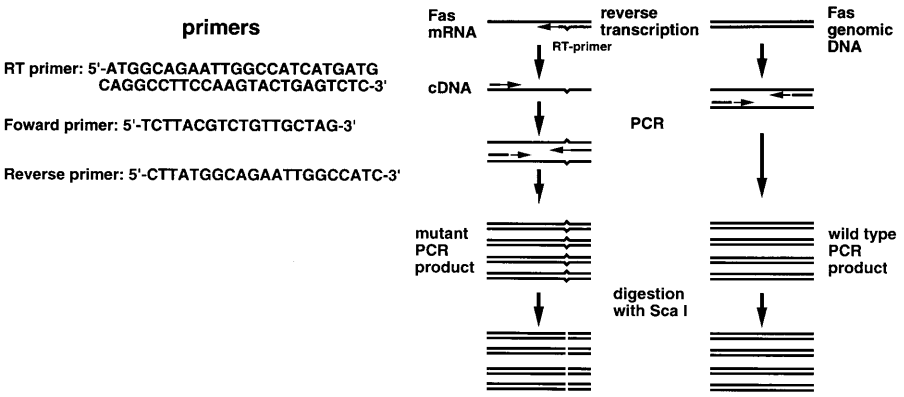

Figure 1. Sequences of primers and strategy of the MRT-PCR. At the RT step, a long reverse primer with a one-base mismatch to the Fas genomic DNA (RT primer) is used, and a point mutation is introduced into the cDNA to create a Sca I restriction site. PCR is performed using a set of primers on the same exon, and the corresponding genomic DNA sequence is coamplified with the cDNA. The reverse primer is identical to the 5′-end of the RT primer. Due to the point mutation, the cDNA is sensitive to Sca I restriction enzyme digestion to which genomic DNA is resistant, and these two amplified fragments are now distinguishable by agarose gel electrophoresis. Genomic DNA is used as control for quantitative mRNA analysis, and amounts of mRNA are expressed as the ratio of PCR products from mRNA and genomic DNA.

stained with propidium iodide using the Cycle Test kit (Becton Dickinson, San Jose, CA, USA) according to the manufacturer's instructions and subjected to FACScan analysis (Becton Dickinson) using CellFit software. Antihuman Fas antibody clone CH-11 was purchased from MBL (Nagoya, Japan).

'Mutagenic' RT-PCR. Total RNA and DNA were isolated from cultured cells by a modified guanidine thiocyanate method [10]. After washing three times with Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline (PBS), 1×10^6 cells were mixed with 1 ml of solution D [4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl and 0.1% 2-mercaptoethanol] [10], 1 ml of neutral buffered phenol, 200 µl of chloroform and 100 µl of sodium acetate (pH 7.4) [9]. Samples were then vortexed for several seconds and centrifuged at 3000 rpm for 40 min. Nucleic acids were precipitated with ethanol (2 ml), and the pellets were rinsed with 70% ethanol and dissolved in 25 µl of TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)). Both RNA and DNA in the samples were totally recovered using this procedure [9].

Fas mRNA was quantitated using 'mutagenic' RT-PCR (MRT-PCR) [9]. We designed three oligonucleotide primers (RT-, forward and reverse primers) based on the sequence of exon 2 of the human *Fas* gene (fig. 1). First, the RT reaction was carried out with 5 µl of nucleic acid samples in 30 µl of reaction mixture containing 200 µM of each dNTP (deoxy nucleoside triphosphate) and $1 \times$ PCR buffer [50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$ and 0.001% (w/v) gelatin] with 5 U of avian reverse transcriptase (Boehringer Mannheim, Mannheim, Germany). At this step, the generation of complementary DNA (cDNA) using the RT primer with a one-base mismatch yielded a new restriction site for the Sca I enzyme (fig. 1). After heat inactivation of the reverse transcriptase at 95 °C for 5 min, forward and reverse primers were added to the reaction at a final concentration of 1.0 µM in addition to 65 µl of $1 \times$ PCR buffer and 2 U of Ampli-Taq polymerase (Perkin Elmer Cetus Corp., Norwalk, CT, USA). Samples were placed in the thermal cycler and subjected to 30 PCR cycles each consisting of 94 °C for 1 min, 62 °C for 2 min and 72 °C for 1.5 min. We then digested 5 µl of PCR products with Sca I enzyme in 20 µl of reaction mixture, and analysed the digested PCR products by electrophoresis in 2% agarose gels.

Southern blotting. Electrophoresed samples were then transferred to a nylon membrane (Schleicher and Schuell, Dassel, Germany), and hybridized with a digoxigenin-labelled (Boehringer Mannheim) 19-mer oligonucleotide probe (CACAAGGGGTAGGCTACGG) which was positioned between the forward and reverse primers. Signals were quantitated using a densitometer, and the amounts of mRNA were expressed as the signal intensity ratio of the PCR products from mRNA to those from genomic DNA as genome equivalents per cell (GEq/cell).

Nuclear staining. For detection of changes in nuclear chromatin, cells were fixed in 1% glutaraldehyde at 4 °C for 30 min. The cell nuclei were stained with Hoechst 33258 (167 µM in PBS, purchased from Wako, Tokyo, Japan) in the dark. After washing in PBS, nuclear samples were mounted on slide glass and visualized under a fluorescence microscope with an ultraviolet (UV) filter.

Results

The MRT-PCR system for *Fas* gene mRNA detection. First, we performed preliminary experiments to determine the optimal concentration of the RT primer, because carryover of the RT primer may result in production of mutated DNA in the subsequent PCR if a high concentration of the RT primer is present [9]. We carried out the MRT-PCR using various concentrations of the RT primer and determined the optimal concentration of the RT primer so that genomic DNA would not be amplified but the yield of cDNA products would be maximal. At concentrations over 200 fmol/10 µl, we observed a faint mutated DNA signal on gels for RT(−) controls. At concentrations below 50 fmol/10 µl, the yields of PCR products were decreased. Therefore, we decided to use the RT primer at a concentration of 100 fmol/10 µl or 10 nM in the subsequent MRT-PCR. We also performed a control experiment to test the effectiveness of our MRT-PCR system. Mutated control DNA was synthesized by adding an excess amount of RT primer at the step of PCR amplification of genomic DNA that was extracted from HeLa cells. Both the wild-type and mutated DNA were purified, quantitated by measuring the optical density at 260 nm. Known concentrations of the purified wild-type and mutated DNA products were mixed and used as PCR templates. The PCR conditions used were identical to those for the MRT-PCR, and 10 nM of the RT primer was used. The ratio of mutated DNA to wild-type DNA of PCR products reflected the ratio of each of these templates in the starting reaction. On the gels, the band at 110 bp was not detected when the template contained no mutation, indicating that the occurrence of new mutations during PCR is negligible using this concentration of RT primer.

Regulation of *Fas* gene expression. To observe the changes in Fas mRNA levels during cell-cycle progression, HeLa cells were plated at a concentration of 1×10^6 cells/10-cm dish and cultivated in DMEM containing 10% FCS for 12 h. Then cells were cultivated in DMEM containing 0.1% FCS for 48 h. This procedure resulted in approximately 90% of the cells being in the quiescent state. The cells commenced growing when they were cultivated in DMEM containing 10% FCS. HeLa cells began to exhibit synchronous cell-cycle progression as described previously [11, 12]. Total RNA and DNA were extracted from cells at 0, 12, 24, 36 and 48 h and subjected to the MRT-PCR for Fas mRNA quantitation. As shown in figure 2, the *Fas* gene mRNA level of the HeLa cells gradually increased after the addition of FCS to the medium, reached a peak at 36 h and returned to the basal level at 48 h. The expression of the *Fas* gene is thus regulated in association with cell-cycle progression in this HeLa cell model system.

***Fas* mRNA levels and induction of apoptosis.** We then determined whether these cells at various stages and with different Fas levels exhibit distinct susceptibility to apoptotic signals. For this purpose, antihuman Fas antibody was added to culture dishes at a concentration of 200 ng/ml at 0, 12, 24, 36 and 48 h after the addition of FCS, and the cells were then cultivated at 38.5 °C for 24 h before harvest. Flow cytometric analysis of harvested cells showed that the fraction undergoing apoptosis (hypodiploid fraction) was $2.2 \pm 2.3\%$ of total cell populations in the cells treated with anti-Fas antibody at 0, 12 and 48 h after the addition of FCS, while it amounted to 17.9 ± 2.3 and 21.5 ± 3.5 (average $\pm$ SE of triplicated experiments) of those in the cells treated at 24 and 36 h, respectively (fig. 2). Nuclear staining with

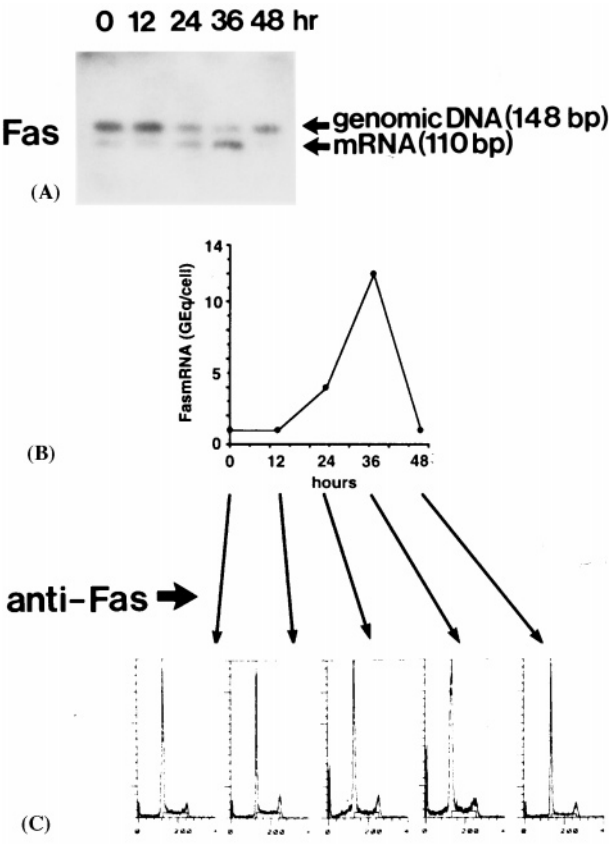

Figure 2. Quantitation of Fas mRNA by MRT-PCR and apoptosis induced by anti-Fas antibody in HeLa cells. The numbers 0, 12, 24, 36 and 48 indicate the time in hours after the addition of FCS to medium. (*A*) Cell-cycle-dependent regulation of Fas gene expression. The smaller band indicates a 110-bp fragment resulting from digestion of the product amplified from Fas mRNA by MRT-PCR. The larger 148-bp band originated from genomic DNA. (*B*) The Fas mRNA levels were expressed as the ratio of the intensity of the mRNA signal to that of the genomic DNA signal (GEq/cell). (*C*) Induction of apoptosis by anti-Fas antibody. HeLa cells at each phase of the cell cycle were treated with anti-Fas antibody for 24 h before harvesting, and subjected to flow cytometric analysis.

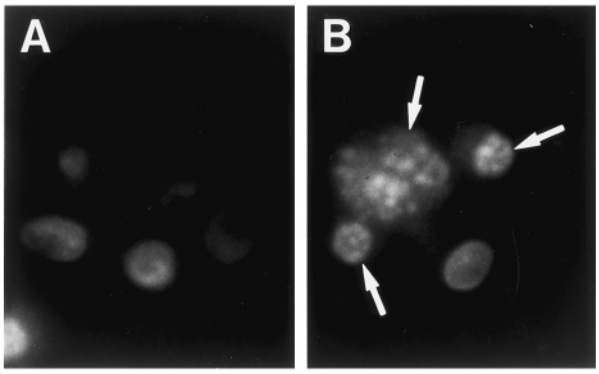

Figure 3. Nuclear morphology of HeLa cells treated with anti-Fas antibody at 0 h (*A*) or 36 h (*B*) after the addition of FCS. The arrows indicate nuclei of HeLa cells fragmented in small round bodies. The stained nuclei were visualized under a fluorescence microscope with a UV filter.

Hoechst 33258 of the HeLa cells treated with anti-Fas antibody at 36 h showed fragmentation of chromatin into small round bodies, characteristic of apoptotic nuclei (fig. 3B). In contrast, cells treated at 0 h did not show signs characteristic of apoptotic morphology (fig. 3A). These results indicate that these HeLa cells with high *Fas* mRNA levels are more susceptible to apoptotic signals than those with low *Fas* mRNA levels.

Discussion

We determined the levels of human *Fas* gene mRNA in HeLa cells by the MRT-PCR which utilizes genomic *Fas* gene DNA as an internal control. We chose a set of primers based on the sequence of exon 2 of the human *Fas* gene, because the cDNA of this exon is sufficiently long so that three primers can be designed based on its sequence to yield PCR product, and it has no restriction site for the Sca I enzyme [1]. This MRT-PCR which allowed us to detect *Fas* gene expression sensitively and specifically has several advantages over conventional methods, including Northern blotting [9]. First, this method is applicable to small amounts of samples. Second, since genomic DNA is used as a quantitative control, the levels of mRNA are expressed as copy number per genome or copy number per cell (the *Fas* genomic DNA is counted as four copies per cell). This exempts us from precisely quantitating the tissue (cell) sample amount, which is an absolute requirement when an extrinsic standard is utilized. However, copy number of mRNA per cell may be underestimated under the condition whereby the rate of replication of genomic DNA is increased, i.e. in the S-phase of the cell cycle. Thus, our estimation of *Fas* mRNA levels at 24 h may be lower than the actual copy numbers.

We determined the levels of human *Fas* gene mRNA in HeLa cells at various stages in cell-cycle progression using the MRT-PCR. The Fas level was low in the quiescent state, but increased as the cell cycle progressed and reached a peak at 36 h after the addition of FCS to DMEM. Although there is a possibility that the addition of FCS to DMEM may have just resulted in the induction of the Fas gene, the peak of *Fas* mRNA levels at 36 h indicates that this is rather late for the Fas gene to be a serum-inducible gene such as c-fos or c-junb [13, 14]. In addition, the promoter region of the human *Fas* gene lacks a serum response element [15]. The addition of anti-Fas antibody to culture medium demonstrated that HeLa cells exhibiting a high level of *Fas* mRNA are more susceptible to apoptotic signals, although the apoptosis-inducing effect of anti-Fas antibody was rather weak, since HeLa cells are relatively resistant to anti-Fas antibody in the absence of pretreatment with interferon-γ [16]. These data indicate that the *Fas* gene product the expression of which is regulated in association with cell-cycle progression is functional in inducing the apoptosis of HeLa cells in response to an apoptotic signal.

The Fas–FasL system is involved in such events as the activation-induced suicide of T cells [3, 4], the deletion of activated B cells [2] or the regulation of apoptosis in the liver [5], and may play an essential role in the pathogenesis of malignancies, autoimmune diseases, insulitis and fulminant hepatitis [2]. Our sensitive system for the detection of *Fas* mRNA may be useful in the elucidation and analysis of the role of the Fas–FasL system in physiological phenomena or pathological disorders.

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