

The Presence of a Short Form of p53 in Chicken Lymphoblastoid Cell Lines during Apoptosis

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ABSTRACT. To examine the roles of a short form of p53 in the regulation of apoptosis in chicken lymphoblastoid tumor cell lines derived from Marek's disease (MD) and avian leukosis (AL), the expressions of the p53 proteins were analyzed in these cell lines in which apoptosis was chemically induced. In MSB1-O derived from MD, the expression of a 40 kDa protein of p53 was decreased and that of a 32 kDa protein, a short form of p53, was increased during apoptosis induced by actinomycin D. In 1104B1 derived from AL, the expressions of 42 and 32 kDa of p53 were increased during the apoptosis. The short form of p53 was undetectable in these cell lines when apoptosis was blocked by the pretreatment with endonuclease inhibitor, Zn²⁺, protease inhibitors, TPCK and TLCK, or caspase inhibitor, Z-VAD-FMK. In the transcriptional level, the expressions of *bcl-2* and *IAP* were decreased in these cell lines during actinomycin D-induced apoptosis, but no change was detected in the expression level of *p53*. These results suggest that, in these chicken tumors, the short form of p53 could play a role in the initiation of apoptosis induced by the chemotherapeutic compound, and that the regulation of its expression may be important for the maintenance of transformation status.

KEY WORDS: apoptosis, Marek's disease, p53.

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The tumor suppressor gene *p53* encodes a transcription factor, p53 [4, 39]. In response to various stimuli, p53 transactivates the expressions of a number of anti-proliferative or pro-apoptotic molecules by its binding to specific DNA sequences, thereby arresting cell cycle, repairing damaged DNA, or inducing apoptosis [8, 11, 12]. The p53 function is regulated by phosphorylation and acetylation of p53 as well as the interaction of p53 with other molecules [5, 27, 33]. By these regulations, p53 selects target promoters of several genes by changing its structure and affinity to bind to the DNA sequences. Mutations in the *p53* genes are the commonest alteration in human and animal cancers [18, 25, 34, 38]. These mutations in the evolutionarily conserved codons, called “hot spot”, are common in diverse types of mammalian cancers, and some mutants lose transcriptional activation potency, and their ability to bind to DNA [19, 20, 30]. Loss of the p53 function, which resulted from several point mutations or inactivation by viral oncoproteins [9], thus lead to tumor formation and loss of the efficiency of chemotherapeutic compounds.

Marek's disease (MD) caused by a herpesvirus, Marek's disease virus (MDV), and avian leukosis (AL) caused by a retrovirus, avian leukosis virus (ALV), are chicken neoplastic diseases [29]. Neither the molecular mechanism(s) of transformation by MDV or ALV nor the roles of p53 on the transformation has been fully understood though p53 can be detected in these tumors [15]. Our previous study showed some abnormalities including point mutations in the *p53*

gene of chicken lymphoblastoid tumor cell lines derived from MD and AL [35]. However, none of these mutations were located at “hot spot”, which has been reported as the site for transformation-activating mutations. Moreover, several deleted forms of the *p53* transcripts were identified in these cell lines [36]. Some of these deletions could cause a frame shift of the encoding p53, possibly resulting in the generation of functionally different p53 molecules. The several deleted forms of *p53* mRNA are detected in MSB1-O and -cl.18 derived from MD throughout the cell cycle or during the apoptosis induced by a chemotherapeutic compound, actinomycin D [37]. In human and murine cell lines, several N- or C-terminally truncated or alternatively spliced isoforms of p53 have been identified [13], and some of them were also shown to regulate p53-dependent apoptosis and cell cycle progression [2, 6, 28, 31]. Thus, the deleted forms of p53 present in MD and AL cell lines could also play a role in the transformation by MDV and ALV through the regulation of apoptosis or cell cycle progression. For these reasons, in this study, to determine whether these deleted isoforms of the *p53* transcripts and proteins are associated with the regulation of apoptosis on MD and AL tumor cell lines, we analyzed the transcripts and proteins expressed in these cell lines treated with either apoptosis inducers, inhibitors or caspase inhibitors.

MATERIALS AND METHODS

Cell lines: MD tumor-derived lymphoblastoid cell line, MDCC-MSB1-O [1], and an AL-derived tumor cell line, LSCC-1104B1 [17] were used in this study. Two cell lines

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were cultured as described previously [35].

Treatments of cells: Cells were treated for 3, 6 and 9 hr with 2 $\mu\text{g}/\text{ml}$ actinomycin D (Wako, Osaka, Japan). In some cases, cells were incubated with 100 μM Z-VAD-FMK (Enzyme Systems Products), Ac-YVAD-CMK (Peptide Institute, Osaka, Japan), Ac-DEVD-CHO (Peptide Institute, Japan), or Ac-LEHD-CHO (Peptide Institute) to inhibit caspase activity, or 0.5 μM Zn^{2+} to inhibit endonuclease, or 100 mM TPCK (Wako) or 500 μM TLCK (Wako) to inhibit protein synthesis.

Detection of apoptosis: Apoptosis was detected by flow cytometric and DNA ladder methods. For flow cytometric analysis, after treated with the chemicals as described, cells were collected and fixed with 70% ethanol at 4°C. After rinsed with PBS, cells were incubated for 30 min with PBS in the presence of RNase A (20 $\mu\text{g}/\text{ml}$), and then stained with propidium iodide (50 $\mu\text{g}/\text{ml}$). The stained cells were analyzed in a flow cytometer (EPICS PROFILE II, Beckman-Coulter, Fullerton, CA).

As apoptotic biomarkers, quantitative measurement of DNA fragmentation and detection of DNA ladder formation were performed. For each assay, approximately 5×10^5 cells were lysed with lysis buffer (50 mM Tris-HCl pH7.8, 10 mM EDTA, 0.5% Triton X-100). The lysates were treated with RNase A (0.4 mg/ml) and Proteinase K (0.2 mg/ml), and resultant samples were analyzed on a 2% agarose gel, and stained with ethidium bromide.

Preparation of total cellular RNA and semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis: Total cellular RNA was isolated from cell lines using the TRIzol reagent (Invitrogen, Gaithersburg, MD) following the manufacturer's instructions. The total cellular RNA (10 mg) was converted to cDNA by reverse transcriptase (RAV-2, Takara, Kyoto, Japan) following the manufacturer's instructions. The cDNA fragments for *p53*, *caspase-3*, *bcl-2*, *IAP*, and *GAPDH* (as a control) cDNA fragments were amplified using sense/antisense primers (Table 1) and *rTaq* polymerase (Takara) at 95°C for 5 min at the initial denaturation step followed by 16 cycles (for *GAPDH*), 26 cycles (for *p53*), 24 cycles (for *caspase-3*), 40 cycles (for *bcl-2*), or 27 cycles (for *IAP*) at 95°C for 30 sec, 50–58°C for 30 sec, 72°C for 30 sec. The resultant PCR

products were analyzed by on a 2% agarose gel, and stained with ethidium bromide.

Western blot analysis: After treated as described above, cells were lysed in 2x SDS buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol), and boiled for 10 min. Samples were separated on 15% SDS-polyacrylamide gels, and transferred to the polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA). The amounts of the samples were adjusted to show equal intensities of bands to one another, determined by a preliminary SDS-PAGE and subsequent staining with Coomassie brilliant blue G-250. The membranes were blocked with PBS containing 5% skim milk and 0.05% Tween 20 at 4°C for overnight, and incubated with either of the two anti-p53 monoclonal antibody (MAb), PAb240 (Cymbus Bioscience, Chilworth, SH, U.S.A.) or HP64 [23] (a gifted from Dr. Soussi), or polyclonal mouse antisera against chicken caspase-3 at room temperature for 1 hr. The membranes were then washed 3 times with PBS containing 0.05% Tween 20 (PBST), and incubated with peroxidase-conjugated goat anti-Mouse IgG (H+L) (Jackson ImmunoResearch Lab. West Grove, PA, U.S.A.). After 3 times washed with PBST, chicken p53 and procaspase-3 proteins were visualized by means of Chemiluminescence Reagent Plus (PerkinElmer, Boston, MA) following manufacturer's instructions.

RESULTS

Comparison of p53 expression in chicken tumor cell lines during apoptosis induced by actinomycin D: To investigate whether the p53 expression in chicken tumor cells is changed or not when apoptosis is induced by actinomycin D, Western blotting was performed using anti-p53 MAbs, PAb240 and HP64, which recognized the epitopes 198–203 (KRHSVV) and 156–171 (EVVRRCPHHERCGGGT) of chicken p53 [23], respectively. By using these MAbs, native chicken p53 is usually detected as proteins with the molecular weight ranging 40–46 kDa, depending on the post-translational modifications on p53 [21, 23]. When MSB1-O and 1104B1 were treated with actinomycin D, DNA fragmentation patterns were observed in both of the cell lines, and the intensities of the laddering were increased in a time-dependent manner (Fig. 1A). Increase in the numbers of apoptotic cells was also confirmed by flow cytometric analysis, and untreated control cell lines did not show any apoptotic features (Fig. 1A). By Western blotting using a MAb, PAb240, 40 (native form of p53) and 32 kDa (short form of p53) proteins were detected in MSB1-O, and 42 kDa (native form of p53) was detected in 1104B1 (Fig. 1B upper). On the other hand, when cells were treated with actinomycin D, the amount of the 40 kDa protein was decreased while that of the short form of p53 was increased in MSB1-O. In 1104B1, the amount of the 42 kDa protein was increased and then slightly decreased at 9 hr after the treatment with actinomycin D, and that of the short form of p53 was increased in a time-dependent manner. Similar results were

Table 1. Primers used to amplify chicken genes in this study

Primer	Nucleotide sequences	Annealing temperature (Numbers of cycles for PCR)
GAPDH-F	5'-TGCAGGTGCTGAGTATGTTGTGGA-3'	58°C
GAPDH-R	5'-CCACAACACGGTTGCTGTATCCAA-3'	(16)
p53-F	5'-GTGGCCGTCTATAAGAAATCAGAG-3'	50°C
p53-R	5'-AAAAAGGGGCGTGGTCAGTCCGA-3'	(26)
caspase-3-F	5'-CAAGAGCCATGATGACAGACAT-3'	55°C
caspase-3-R	5'-CACACTGTTTCATCTGGTCCAC-3'	(24)
bcl2-F	5'-CAACTGGATCCAGGACAA-3'	52°C
bcl2-R	5'-CGATAAACTGGGTGACTCTA-3'	(40)
IAP-F	5'-ATGGCTGAAGATGAGAAGAGG-3'	54°C
IAP-R	5'-TGCAAGTTCCTCTTATGA-3'	(27)

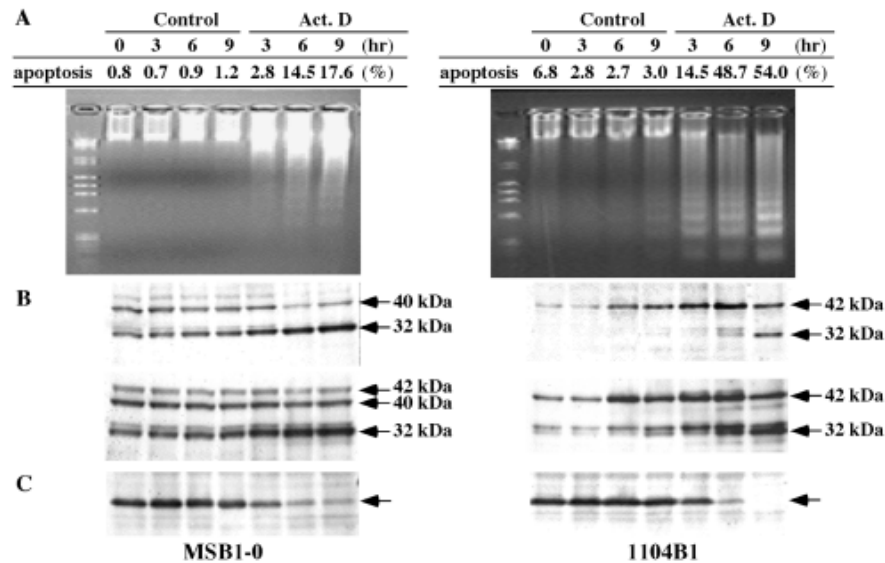


Fig. 1. The presence of short forms of the p53 proteins in tumor cell lines derived from Marek's disease (MSB1-O) and avian leucosis (1104B1) even when apoptosis was induced. MSB1-O and 1104B1 cell lines were treated with actinomycin D (Act. D) for 3, 6 and 9 hr, and then apoptosis of the cells was analyzed by flow cytometry and DNA fragmentation assay (A). Percentages of cells undergoing apoptosis were shown. Time course changes in the p53 expression in MSB1-O and 1104B1 after the treatment with actinomycin D (B). Cell lysates were analyzed by Western blotting using anti-p53 MAbs, PAb240 (upper) and HP64 (lower). Expression of procaspase-3 in these cell lines after the treatment with actinomycin D were also analyzed using anti-chicken caspase-3 polyclonal antibody (C, arrows).

obtained when the other MAb, HP64, was used (Fig. 1B lower). The expression of procaspase-3 was decreased in both MSB1-O and 1104B1 after 6 hr of the exposure to actinomycin D (Fig. 1C).

The increased expression of the short form of p53 coincides with the induction of apoptosis: Since the amount of the short form of p53 was increased during apoptosis, the short form of p53 may regulate the apoptosis-inducing ability of p53. Therefore, the expression of p53 was examined in MSB1-O and 1104B1 when these cell lines were pretreated with either apoptosis inhibitors including Zn^{2+} (Fig. 2), TPCK, TLCK (Fig. 3) or caspase inhibitors (Fig. 4), and then treated with actinomycin D. DNA fragmentation was not detected in the cell lines pretreated with these inhibitors (Figs. 2A, 3A and 4A), indicating that actinomycin D-induced apoptosis was blocked in these cell lines by these pretreatments. When the expressions of p53 and procaspase-3 were analyzed by Western blotting (Figs. 2A, 3A and 4A), neither increase in the amount of the short form of p53 (32 kDa) nor decrease in that of procaspase-3 was observed. On the other hand, the 40 and 42 kDa proteins were constantly detected in inhibitor-treated MSB1-O and 1104B1, respectively. However, the short form of p53 found in the control of Fig. 1 was not detected in these experiments (Figs. 2B, 3B and 4B), and this was due to the difference in the lots of PAb240 used in these experiments. Apoptosis was not blocked in these cell lines pretreated with caspase inhibitors, a caspase-1/-4 inhibitor, Ac-YVAD-CMK, caspase-3/-6/-7/-10 inhibitor, Ac-DEVD-CHO, or caspase-9 inhibitor, Ac-LEHD-CHO, and thus, the

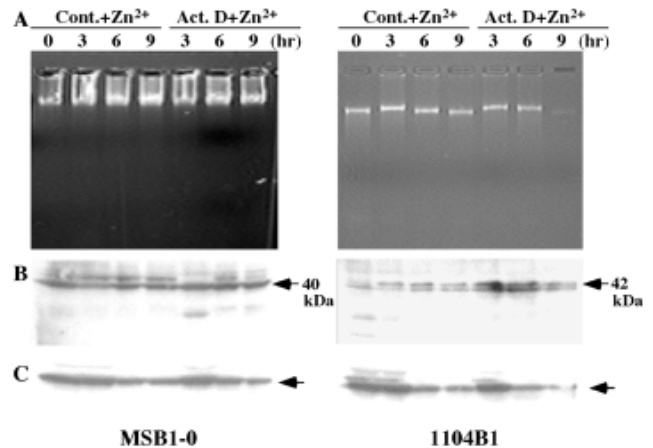


Fig. 2. The expression of the p53 proteins in MSB1-O and 1104B1 when actinomycin D-induced apoptosis was inhibited by endonuclease inhibitor, Zn^{2+} . DNA fragmentation was assayed in cells which were treated with Zn^{2+} , and then treated with actinomycin D (A). Expressions of p53 (B) and procaspase-3 (C, arrows) in these cells were analyzed by Western blotting using anti-p53 MAb, PAb240, and anti-chicken caspase-3 polyclonal antibody, respectively.

increased expression of the short form of p53 was detected.

Expression of apoptosis-related gene with or without apoptosis: To determine whether the expressions of apoptosis-related genes are altered in MSB1-O and 1104B1 either when apoptosis was induced by actinomycin D or when actinomycin D-induced apoptosis is blocked by Zn^{2+} , RT-PCR analysis was performed (Fig. 5). In MSB1-O and 1104B1

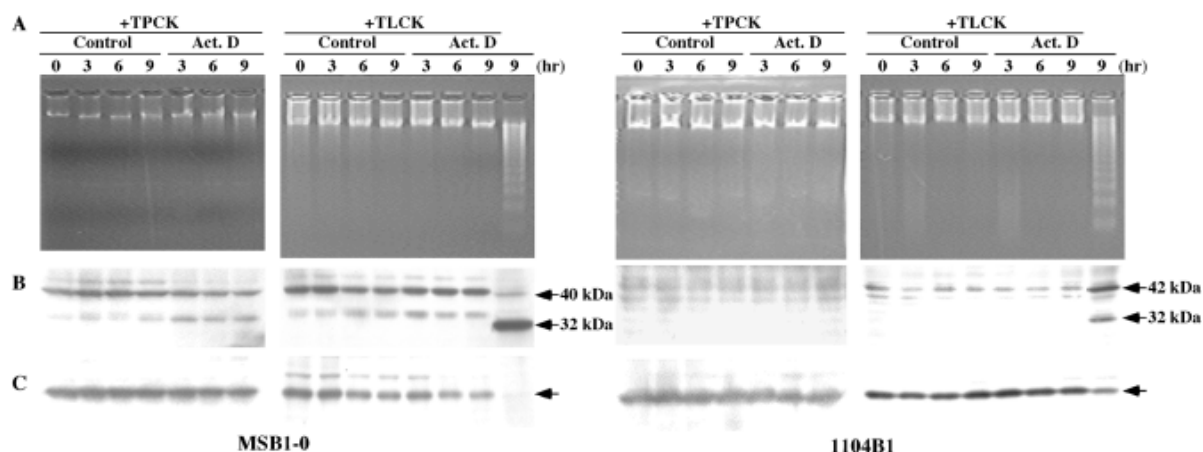


Fig. 3. The expression of the p53 proteins in MSB1-O and 1104B1 when actinomycin D-induced apoptosis was inhibited by protein synthesis inhibitors, TPCK and TLCK. DNA fragmentation was assayed in cells which were treated with either TPCK or TLCK, and then treated with actinomycin D (A). Expressions of p53 (B) and procaspase-3 (C, arrows) in these cells were analyzed by Western blotting using anti-p53 MAb, PAb240, and anti-chicken caspase-3 polyclonal antibody, respectively.

treated with actinomycin D, the expressions of the *bcl-2*, *IAP* and *caspase-3* genes were reduced compared to those in untreated cell lines, while no significant change was observed in the expression of the *p53* gene. When these cell lines were pretreated with Zn^{2+} , on the other hand, the treatment with actinomycin D did not alter the expressions of these genes including the *p53* gene. Exceptions were the *IAP* expression, which was undetectable in MSB1-O after 3 to 6 hr of the exposure to actinomycin D, and the *bcl-2* expression, which was slightly decreased as these cell lines underwent apoptosis.

DISCUSSION

Loss of the p53 function, that resulted from several point mutations or deletions, in human and animal cancer leads to oncogenesis and loss of the efficient anti-cancer effects by chemotherapeutic compounds [34]. In this study, it was shown that the expression of a short form of p53 (32kDa) was increased during apoptosis of chicken lymphoblastoid cell lines, MSB1-O and 1104B1, induced by a chemotherapeutic compound, actinomycin D (Fig. 1). Furthermore, a short form of p53 was not detected when apoptosis was inhibited in these cells (Figs. 2, 3 and 4). Since no significant change was observed in the *p53* transcription in the MD and AL cell lines even after the induction of apoptosis (Fig. 5), the short form of p53 might be resulted either from decomposition of p53 by cellular proteases, or alternative splicing of the *p53* gene which was not detected in RT-PCR analysis used in this study.

Several alternatively spliced murine and human p53 transcripts that encode p53 proteins with altered N- or C-terminus have been reported [3, 13, 14, 16, 22, 31]. The functions of these alternatively spliced p53 isoforms include the regulation of p53-dependent apoptosis [2], and the control of ubiquitination, cell localization, and activity of p53 [14]. In addition, the overexpression of murine p44, a short p53 iso-

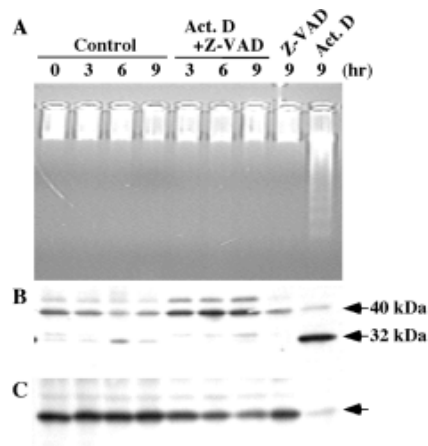


Fig. 4. The expression of the p53 proteins in MSB1-O when actinomycin D-induced apoptosis was inhibited by caspase inhibitor, Z-VAD-FMK. DNA fragmentation was assayed in cells which were treated with Z-VAD-FMK, and then treated with actinomycin D (A). Expressions of p53 (B) and procaspase-3 (C, arrows) in these cells were analyzed by Western blotting using anti-p53 MAb, PAb240, and anti-chicken caspase-3 polyclonal antibody, respectively.

form which lacks a part of the transactivation domain at the N-terminus, results in p53-dependent cellular aging and reduced life span in transgenic mouse [24]. Truncated murine p44 can be tumorigenic in mice through its ability to impair the p53 activity [26, 32]. These observations suggested that the quantitative and qualitative balances between p44 and p53 could be important to control the p53 functions. Similarly, the short form of p53 found in this study could also regulate the apoptosis through modulating the p53 activities in chicken tumor cells. However, the spliced variants of the p53 transcripts detected by RT-PCR in these chicken tumor cell lines were completely different from those reported previously: most of them lack exons 4–8 cor-

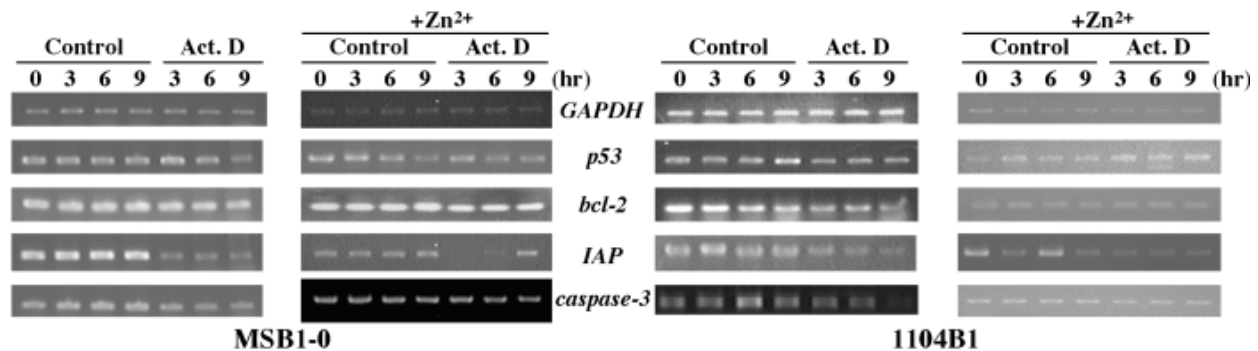


Fig. 5. RT-PCR analysis of apoptosis-related genes expressed in tumor cell lines when apoptosis was induced or inhibited. RNA samples were prepared from cells treated with either actinomycin D alone or Zn^{2+} and actinomycin D. Resultant PCR products were resolved on agarose gels, and analyzed by staining with ethidium bromide.

responding to regions encoding both the SH3 and DNA-binding domains, and these deletions caused frame shifts in many of these variants [36]. Additional studies are necessary to determine whether a spliced variant which can encode the short form of p53 are present in these cell lines.

The short form of p53 could be potentially produced by a mechanism other than the selective increase in the alternative splicing. It has been reported that p53-mediated apoptosis does not require transcriptional activation, because inhibition of transcription did not affect p53-dependent apoptosis [7]. In chicken tumor cells, when apoptosis was induced, although the expression of the short form of p53 was increased, the amount of the p53 transcripts was not changed. These results suggest that p53- or the short form of p53-mediated apoptosis does not also require transcriptional activation of the p53 gene. It has been shown that the interaction of p53 with the site of DNA damage, which can trigger the apoptosis-related signaling pathway, induces selective proteolytic cleavage of p53, resulting in the production of p53 which lacks N- and/or C-terminal domains [28]. These cleaved fragments still retain the function of sequence-specific DNA binding. In this study, the short form of p53 was detected by MAbs, PAb240 and HP64, both of which recognize the epitopes in the DNA binding domain [23], suggesting the possibility that the short form of p53 is N-, and/or C-terminally deleted, and that it also retain the functions of p53 activity such as sequence-specific DNA binding. Since the association of the ribosomal proteins with MDM2 is responsible for the p53 activation after the treatment with actinomycin D [10], MDM2 may regulate the expression of the short form p53. The molecular size of p53 detected in MSB1-O was slightly different from that in 1104B1. The p53 with a molecular weight of 42 kDa found in 1104B1 was also detected in other MD-, AL- and RE-derived tumor cell lines [36], suggesting that this molecule is expressed as the most dominant form of the native p53. On the other hand, the 40 kDa protein detected only in MSB1-O could be either a structurally altered or deleted form of the native p53, that is caused by MDV.

Thus, it is now clear that multiple forms of p53 including the 32 kDa short form are present in chicken tumor cell

lines, though the functions or biological significance of these p53 isoforms are still unknown. In this study, it was suggested that the short form of p53 in chicken tumor cells was closely related to the regulation of apoptosis. Since the regulation of the functions of the short form of p53 might be associated with the mechanisms for the growth of tumor cells and maintenance of transformation status, it would be necessary to study the function(s) of the short form of p53 in chicken tumors. In addition, the induction of apoptosis in MSB1-O and 1104B1 by actinomycin D was blocked by Z-VAD-FMK, a broad-spectrum inhibitor of caspases, but not by caspase-1, 3, 4, 6, 7, 9 and 10 inhibitors. These results show that unknown caspase pathways may be present or that these caspase inhibitors do not function as regulators of apoptosis in chickens. Unlike mammalian species, it has not been studied in detail on the functions of p53, caspases and apoptosis-related proteins in chicken tumor cells, and thus, it will be also necessary to study the functions of these proteins with the relation to p53 to further clarify the transformation process by chicken oncogenic viruses.

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