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Apoptosis and p53 expression in rat adjuvant arthritis

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Statement of findings

The kinetics of apoptosis and the apoptosis-regulating gene *p53* in adjuvant arthritis (AA) were investigated to assess the value of the AA rat model for testing apoptosis-inducing therapies. Very few terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL)-positive cells were detected during the early phases of AA, but on day 23 (chronic arthritis) the percentage of TUNEL-positive cells was significantly increased. Expression of *p53* in synovial tissue gradually increased from days 5–23, which was markedly higher than *p53* levels in rheumatoid arthritis (RA) synovium. Significant apoptosis only occurs late in rat AA and is concordant with marked *p53* overexpression, making it a useful model for testing proapoptotic therapies, but rat AA is not the best model for *p53* gene therapy because dramatic *p53* overexpression occurs in the latter stages of the disease.

Keywords: adjuvant arthritis, apoptosis, *p53*, rheumatoid arthritis

Synopsis

Introduction: RA is a chronic inflammatory disorder that is characterized by inflammation and proliferation of synovial tissue. The amount of DNA fragmentation is significantly increased in rheumatoid synovium. Only low numbers of apoptotic cells are present in rheumatoid synovial tissue, however. The proportion of cells with DNA strand breaks is so great that this disparity suggests impaired apoptosis. Therefore, the development of novel therapeutic strategies that are aimed at inducing apoptosis in rheumatoid synovial tissue is an attractive goal.

Although animal models for arthritis only approximate RA, they provide a useful test system for the evaluation of apoptosis-inducing therapies. AA in rats is among the most commonly used animal models for RA. For the interpretation of such studies, it is essential to characterize the extent to which apoptosis occurs during the natural course of the disease.

Therefore, we evaluated the number of apoptotic cells and the expression of *p53* in various phases of AA.

Materials and methods: In order to generate the AA rat model, Lewis rats were immunized with *Mycobacterium tuberculosis* in mineral oil on day 0. Paw swelling usually started around day 10. For the temporal analysis rats were sacrificed on days 0, 5 (prearthritis), 11 (onset of arthritis), 17 (accelerating arthritis), or 23 (chronic arthritis).

For the detection of apoptotic cells, the hind paws were harvested on days 0 ($n=6$), 5 ($n=6$), 11 ($n=6$), 17 ($n=6$), or 23 ($n=4$). The right ankle joints were fixed in formalin, decalcified in ethylenediaminetetra-acetic acid, embedded in paraffin, and sectioned. The TUNEL method was applied. The percentage of TUNEL-positive cells of the total inflammatory cell infiltrate was noted.

For Western blot analysis, hind paws were harvested on days 0 ($n=2$), 5 ($n=3$), 11 ($n=4$), 17 ($n=4$), or 23 ($n=4$). In addition, hind paws of normal rats ($n=2$) were studied. The right ankle joints were snap frozen and pulverized. Synovial tissue was also obtained by arthroscopy of three patients with longstanding (>5 years) RA. After protein extraction in lysis buffer, equal amounts of protein samples from lysates were pooled and examined by Western blot analysis using anti-p53 monoclonal antibody DO7, which recognizes wild-type and mutant p53 from rodents and humans.

For immunohistochemical analysis, six rats were sacrificed on day 23 after immunization and synovial tissue of the right ankle joints was snap frozen and evaluated by immunohistochemistry using anti-p53-pan. The sections were evaluated semi-quantitatively using a 0–4 scale.

The Kruskal–Wallis test for several group means was used to compare the percentage of TUNEL-positive cells at different time points.

Results: The percentages of TUNEL-positive cells were strongly dependent on the stage of the disease. Very few TUNEL-positive cells were detected in normal rats or in the early phases of AA; the number of TUNEL-positive cells was 1% or less of the total cell infiltrate, including neutrophils, from days 0–17 (Table 1). On day 23, however, the percentage of TUNEL-positive cells was significantly increased [$15.8 \pm 5.1\%$ (mean \pm standard error of the mean); $P=0.01$]. TUNEL-positive cells were observed in the intimal lining layer and synovial sublining of the invasive front, as well as in the articular cartilage (Fig. 1). Subsequently, we examined expression of the tumor suppressor gene p53, because this is a key regulator of apoptosis. Expression of p53 in pooled rat AA joint extracts

Table 1

Percentages of TUNEL-positive cells and p53 expression in synovial tissue of rats with adjuvant arthritis in different phases of the disease

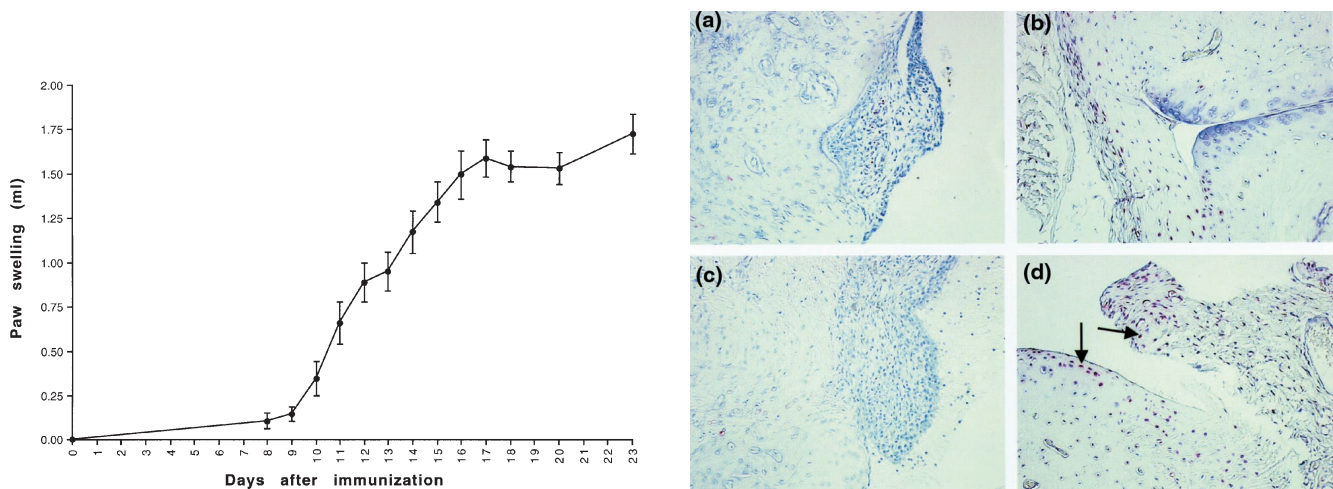
Day	TUNEL-positive cells (%)	Expression of p53 (arbitrary units)
0	0.2 \pm 0.2	6
5	0.3 \pm 0.2	13
11	0.5 \pm 0.2	98
17	1.0 \pm 0.0	153
23	15.8 \pm 5.1	173

Values are expressed as mean \pm standard error of the mean percentages of TUNEL-positive cells in synovial tissue, or as p53 expression in pooled samples of rat joints in arbitrary units.

gradually increased from day 0 (6 arbitrary units) to day 23 (173 arbitrary units), which was markedly higher than p53 levels in RA synovium (32 arbitrary units; Table 1). Overexpression of p53 protein on day 23 was confirmed by immunohistochemistry in a separate experiment in six rats with AA. Overexpression of p53 was observed in the intimal lining layer and synovial sublining in all rats on day 23. In all cases a semiquantitative score of 4 was assigned, indicating that 51% or more of the cells were positive, whereas control sections were negative.

Discussion: The results presented here reveal that the number of TUNEL-positive cells remained very low until chronic arthritis developed. This indicates that, although there was sufficient

Figure 1



(Left) Swelling of the left hind paws (mean \pm standard error of the mean) in different phases of adjuvant arthritis (AA). Swelling usually starts around day 10 after immunization, followed by the development of accelerating arthritis (day 17) and chronic arthritis (day 23). **(Right)** Distribution of TUNEL-positive cells (red, indicated by arrows) in joints of AA rats on day 11 (**a and c**) and on day 23 (**b and d**). Very few TUNEL-positive cells were detected in normal rats or during the onset of arthritis, whereas the number of number of TUNEL-positive cells was significantly increased in the synovium (and also in cartilage) of rats with chronic AA. TUNEL method counterstained with Mayer’s hemalum. Original magnification 250 \times .

DNA damage to cause an increment in p53 expression in the early phases, DNA strand breaks that can be detected by TUNEL assays only occurred in chronic AA. The observation that TUNEL-positive cells were nearly absent in early AA clearly indicates that only very few cells were undergoing programmed cell death. This is an important observation, which makes it possible to study the effects of apoptosis-inducing therapies *in situ* in early and accelerating AA. An effective therapy would obviously increase the number of TUNEL-positive cells.

There is already some overexpression of p53 in the preclinical phase and during the onset of the arthritis, with an additional increment in p53 expression during accelerating and chronic arthritis. Presumably, this is wild-type p53, because the disease duration is likely too short to allow for the development of p53 mutations. Transcription of p53 is probably increased in response to the toxic environment of the inflamed joint. The

increased expression of p53 in the joints of rats with chronic AA was even greater than that observed in synovial tissue of RA patients with long-standing disease.

Overexpression of p53 and increased numbers of apoptotic cells did not occur simultaneously in this model; rather p53 overexpression preceded increased apoptosis. Activation of p53 leads to induction of cell growth arrest, allowing time for DNA repair. It appears that DNA damage is only extensive enough to induce apoptosis in the latter stages of AA. Factors other than p53 may also play an important role in the actual induction of apoptosis.

Taken together, significant apoptosis only occurs late in AA and it follows marked p53 overexpression, making it a useful model for testing proapoptotic therapies. AA is not the best model for p53 gene therapy, however, because dramatic p53 overexpression occurs in the latter stages of the disease.

Full article

Introduction

RA is a chronic inflammatory disorder that is characterized by inflammation and proliferation of synovial tissue. The disease is still associated with long-term morbidity and early mortality, despite treatment with antirheumatic drugs. Inadequate apoptosis appears to contribute toward prolonged survival and constitutive activation of specialized cells in rheumatoid synovium [1,2]. The amount of DNA fragmentation is significantly increased in rheumatoid synovium [3,4], which is presumably due to the toxic environment of the chronically inflamed joint [5]. Only low numbers of apoptotic cells are present in rheumatoid synovial tissue, however [4,6–8]. The proportion of cells with DNA strand breaks is so great that this disparity suggests impaired apoptosis. The observation that mice with the lymphoproliferative or generalized lymphoproliferative disorder, which have mutations that inactivate Fas and Fas ligand, respectively, develop pathology similar to that observed in immune-mediated diseases [9,10] illustrates that reduced apoptosis may play an important role in the pathogenesis of synovitis.

The p53 tumor suppressor is a key regulator of DNA repair and cell replication [11]. DNA damage activates p53, thereby inducing cell growth arrest to allow time for DNA repair. When DNA damage is extensive the cells may undergo apoptosis. Inactivation of the p53 gene renders cells less susceptible to undergo apoptosis [12]. The p53 system ensures that cells with damaged DNA either die or are repaired. We have previously proposed that impaired apoptosis in rheumatoid synovial tissue might be explained in part by the development of permanent genetic changes in the p53 tumor suppressor gene [5,13]. In addition,

other factors may be involved, such as protection against apoptosis by nuclear factor- κ B activation [14–16], a relative deficiency of functional Fas ligand in the RA joint [17], and expression of antiapoptotic molecules, such as bcl-2 [3] and sentrin [18]. Therefore, the development of novel therapeutic strategies aimed at inducing apoptosis in rheumatoid synovial tissue is an attractive goal.

Although animal models of arthritis only approximate RA, they provide a useful test system for the evaluation of apoptosis-inducing therapies. AA in rats is among the most commonly used animal models for RA [19,20]. This model has recently been used to investigate the effects of bisindolylmaleimide, a compound that facilitates Fas-mediated apoptosis [21]. Rat AA might also provide a useful screening model for the evaluation of gene therapies that are aimed at induction of apoptosis, because the size of the joints permits relatively easy intra-articular injection [22]. For the interpretation of such studies, however, it is essential to characterize the extent to which apoptosis occurs during the natural course of the disease. Therefore, we evaluated the number of apoptotic cells and the expression of p53 in various phases of AA.

Materials and methods

Adjuvant arthritis model

Male Lewis rats (150–200 g) were immunized at the base of the tail with 1 mg *Mycobacterium tuberculosis* H37 RA (Difco, Detroit, MI, USA) in 0.1 ml mineral oil on day 0 [23]. Paw swelling usually started around day 10. For the temporal analyses, rats were killed on days 0, 5 (prearthritis), 11 (onset of arthritis), 17 (accelerating arthritis), or 23 (chronic arthritis) by carbon dioxide narcosis. All animals

were handled in accordance with University of California San Diego Animal Subjects Committee and United States Department of Agriculture guidelines.

Detection of apoptotic cells

The hind paws were harvested on days 0 ($n=6$), 5 ($n=6$), 11 ($n=6$), 17 ($n=6$), or 23 ($n=4$). The right ankle joints were fixed in formalin, decalcified for 4 weeks in 15% ethylenediaminetetra-acetic acid in phosphate-buffered saline, embedded in paraffin, and sectioned. For detection of apoptotic cells the TUNEL method was applied, based on terminal deoxynucleotidyl transferase-mediated labeling of free 3'-hydroxy termini exposed in cells that exhibit DNA strand breaks. An *in situ* cell death detection alkaline phosphatase kit from Boehringer Mannheim (Indianapolis, IN, USA) was used according to the manufacturer's instructions. For detection of alkaline phosphatase activity we used the alkaline phosphatase substrate kit I (Fast Red) from Vector Laboratories (Burlingame, CA, USA). The percentage of TUNEL-positive cells of the total inflammatory cell infiltrate was noted.

Western blot analysis

Hind paws were harvested on days 0 ($n=2$), 5 ($n=3$), 11 ($n=4$), 17 ($n=4$), or 23 ($n=4$). In addition, hind paws of normal rats ($n=2$) were studied. After removal of skin and muscle tissue, the right ankle joints were snap frozen in liquid nitrogen and pulverized. Synovial tissue was also obtained by arthroscopy of three patients with longstanding (>5 years) rheumatoid factor-positive, erosive RA; these patients have been described previously [24]. All patients had active arthritis in a knee joint and elevated serum levels of C-reactive protein. The patients were treated with nonsteroidal anti-inflammatory drugs. None were treated with corticosteroids or immunosuppressive drugs, such as azathioprine, methotrexate, or cyclophosphamide, within 3 months before study entry [24]. After protein extraction in lysis buffer, equal amounts of protein samples (in total 20 $\mu\text{g}/\text{lane}$) from lysates were pooled and run on a gel in order to normalize for differences in synovial cellularity [24]. The pooled samples were then transferred onto a nitrocellulose membrane, and p53 protein detected with 0.25 $\mu\text{g}/\text{ml}$ of the Immunoglobulin G_{2b} mouse anti-p53 monoclonal antibody DO7 (Novocastra Laboratories Ltd, Newcastle, UK), which recognizes wild-type and mutant p53 from rodents and humans. After incubation with horseradish peroxidase-conjugated goat-antimouse antibody, horseradish peroxidase activity was detected using hydrogen peroxide as the substrate and visualized by chemiluminescence. Densitometry was performed with Image software version 1.57 (National Institutes of Health, Bethesda, MD, USA). Results are expressed as arbitrary densitometry units.

Immunohistochemistry

Six rats were sacrificed on day 23 after immunization, and synovial tissue of the right ankle joints was snap frozen in

Tissue-Tek OCT (Miles Diagnostics, Elkhart, IN, USA) by immersion in methylbutane (-70°C). All slides were stained in one procedure. Endogenous peroxidase activity was inhibited using 0.1% sodium azide and 0.3% hydrogen peroxide in phosphate-buffered saline for 30 min. The biotinylated anti-p53-pan (Boehringer Mannheim) was diluted to a final concentration of 2 $\mu\text{g}/\text{ml}$ and incubated for 60 min. In negative control sections the primary antibody was omitted or irrelevant antibody was applied at the same concentration as the primary antibody. This was followed by incubation with avidin-biotin-peroxidase complex (Vectastain ABC Kit; Vector Laboratories), biotinylated tyramine, and horseradish peroxidase-conjugated streptavidin, as previously described [24]. Horseradish peroxidase activity was detected using hydrogen peroxide as substrate and 3,3'-diaminobenzidine (DAB; Vector Laboratories) as dye. Sections were coded and randomly analyzed [24]. The sections were evaluated semiquantitatively using a 0–4 scale as follows: 0, no staining; 1, rare positive staining or trace staining (1–5%); 2, scattered clusters of positive cells (6–15%); 3, moderate staining in a specific region (16–50%); and 4, extensive staining throughout a region (51–100%) [24].

Statistical analysis

The Kruskal-Wallis test for several group means was used to compare the percentage of TUNEL-positive cells at different time points.

Results

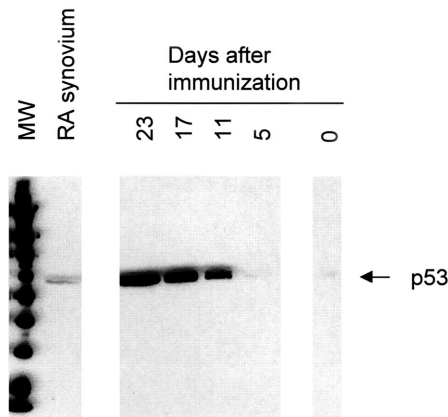
Apoptosis in different phases of adjuvant arthritis

Detection of apoptotic cells was performed on the basis of *in situ* labeling of DNA strand breaks. Representative examples of the TUNEL stainings in relation to the paw volumes in various phases of the disease are shown in Figure 1. The percentages of TUNEL-positive cells were strongly dependent on the stage of the disease. Very few TUNEL-positive cells were detected in normal rats or during the early phases of AA; the number of TUNEL-positive cells was 1% or less of the total cell infiltrate, including neutrophils, from days 0–17 (Table 1). On day 23, however, the percentage of TUNEL-positive cells was significantly increased ($15.8 \pm 5.1\%$ [mean \pm standard error of the mean]; $P=0.01$). TUNEL-positive cells were observed in the intimal lining layer and synovial sublining of the invasive front as well as in the articular cartilage (Fig. 1).

Expression of p53 in different phases of adjuvant arthritis

Subsequently, we examined expression of the tumor suppressor gene p53, because this is a key regulator of apoptosis. Expression of p53 in pooled rat AA joint extracts gradually increased from day 0 (6 arbitrary units) to day 23 (173 arbitrary units), which was markedly higher than p53 levels in RA synovium (32 arbitrary units; Table 1 and Fig. 2). Overexpression of p53 protein on day 23 was confirmed by immunohistochemistry in a separate experiment

Figure 2



Western blot analysis showing immunoreactive *p53* in pooled joint extracts of rats with AA and pooled synovial tissue samples of patients with RA. Expression of *p53* gradually increased from days 0–23 in rat AA (see also Table 1). Overexpression on day 23 (173 arbitrary units) was markedly higher than *p53* levels in RA synovium (32 arbitrary units).

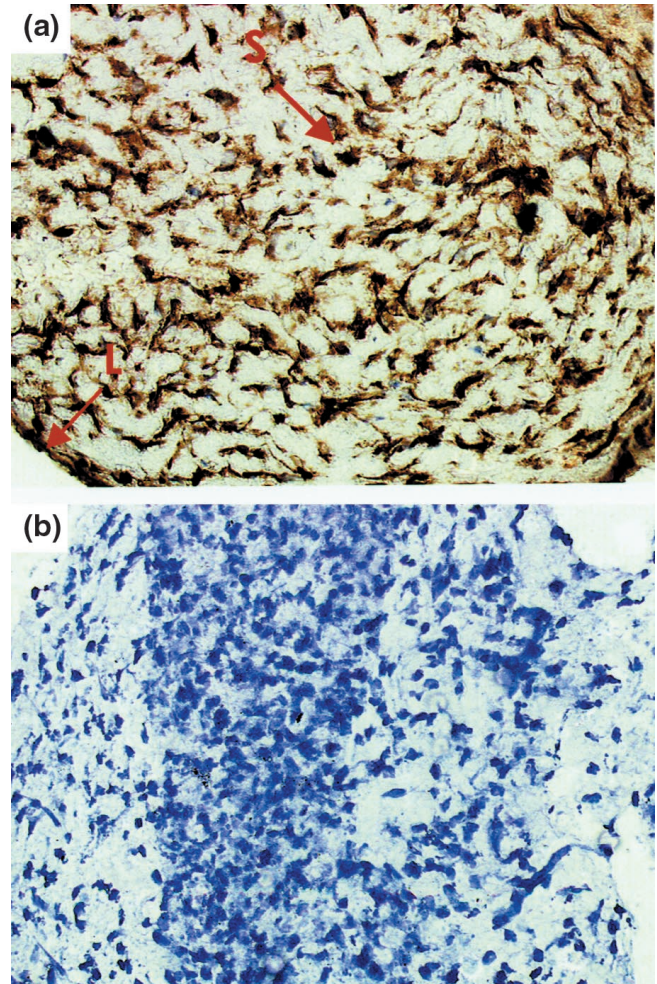
in six rats with AA. Overexpression of *p53* was observed in the intimal lining layer and synovial sublining in all rats on day 23 (Fig. 3). In all cases a semiquantitative score of 4 was assigned, indicating that 51% or more of the cells were positive, whereas control sections were negative.

Discussion

Rat AA is a T-cell dependent disease, which is characterized by paw swelling, joint erosions and ankylosis, as well as systemic manifestations. Infiltration of the synovium by leukocytes precedes the development of clinical signs and symptoms of arthritis [25,26]. There is also an increase in the numbers of CD8⁺ T cells and B cells in the regional lymph nodes in the preclinical phase [27]. As shown in the present study, clinical signs of arthritis usually appear by days 10–12. Subsequently, paw volume markedly increases as a result of cellular infiltration and edema of synovial tissue [28].

The results presented here reveal that the number of TUNEL-positive cells remained very low until chronic arthritis developed. Severe disease and marked paw swelling characterize this phase. The results indicate that, although there was sufficient DNA damage to cause an increment in *p53* expression in the early phases, DNA strand breaks that can be detected by TUNEL assays only occurred in chronic AA. In general, the results may be false positive, because TUNEL-positive cells are not necessarily apoptotic [2]. The observation that TUNEL-positive cells were nearly absent in early AA, however, clearly indicates that only very few cells were undergoing programmed cell death. We have recently shown that there is increased oxidative stress in chronic arthritis [29], which

Figure 3



Representative synovial tissue from a rat with adjuvant arthritis on day 23, showing marked *p53* overexpression [(a), indicated by arrows]. Both cytoplasmic and nuclear staining was noted in the intimal lining layer (L) and in the synovial sublining (S). Staining was absent in the negative control section (b). Monostaining peroxidase technique with tyramine enhancement counterstained with Mayer's hemalum. Original magnification 400 \times .

may play a pivotal role in the induction of DNA strand breaks [5]. The results in the AA model suggest that the production of reactive oxygen and nitrogen species is not sufficiently increased in the earliest phases of arthritis to lead to induction of apoptosis. This is an important observation, which makes it possible to study the effects of apoptosis-inducing therapies *in situ* in early and accelerating AA. An effective therapy would obviously increase the number of TUNEL-positive cells.

There is already some overexpression of *p53* in the preclinical phase and during the onset of the arthritis, with an additional increment in *p53* expression during accelerating and chronic arthritis. Presumably, this is wild-type *p53*,

because the disease duration is probably too short to allow for the development of *p53* mutations. Transcription of *p53* is probably increased in response to the toxic environment of the inflamed joint, with local production of oxygen radicals, nitric oxide [30], and cytokines. Similarly, exposure of human fibroblasts to nitric oxide generated from a nitric oxide donor or from overexpression of inducible nitric oxide synthase may result in accumulation of wild-type *p53* [31]. There are also several examples of overexpression of wild-type *p53* in human inflammatory diseases, which include the following: inflammation in atherosclerotic plaques [32], idiopathic pulmonary fibrosis [33], *Helicobacter pylori*-associated gastritis [34,35], ulcerative colitis [36], Crohn's disease [36], chronic pancreatitis [37], infectious colitis [38], lymphocytic thyroiditis [39], and RA [1,24,40,41]. The increased expression of *p53* in the joints of rats with chronic AA was even greater than that observed in synovial tissue of RA patients with long-standing disease. Because there is already dramatic *p53* overexpression in the latter stages of the disease, AA is probably not the best model to evaluate *p53* gene therapy. Indeed, we could achieve only a marginal additional increase in *p53* expression by *in vivo* gene transfer (unpublished data).

Overexpression of *p53* and increased numbers of apoptotic cells did not occur simultaneously in this model; rather *p53* overexpression preceded increased apoptosis. Activation of *p53* leads to induction of cell growth arrest, allowing time for DNA repair. It appears that DNA damage is only extensive enough to induce apoptosis in the latter stages of AA. Factors other than *p53* may also play an important role in the actual induction of apoptosis, such as tumor necrosis factor- α , interactions between Fas and Fas ligand, and degranulation of granules that contain granzymes and perforin.

Taken together, significant apoptosis only occurs late in AA and it follows marked *p53* overexpression, making it a useful model for testing proapoptotic therapies. AA is not the best model for *p53* gene therapy, however, because dramatic *p53* overexpression occurs in the latter stages of the disease.

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