

High expression of p21^{Waf1} in sarcoid granulomas: a putative role for long-lasting inflammation

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Abstract: In sarcoid granulomas, apoptotic events are reduced, which explains their characteristic long-lasting inflammation. We have described that interferon- γ (IFN- γ) inhibits apoptosis in macrophages through the expression of p21^{Waf1}. Here, we explore the molecular mechanisms involved in the inhibition of apoptosis in sarcoid granulomas. We analyzed skin biopsies from 19 sarcoidosis patients and 16 controls. Total RNA was subjected to semiquantitative reverse transcriptase-polymerase chain reaction analysis. There was no difference found in the expression of proapoptotic (Bax and Bcl-X_s) or antiapoptotic (Bcl-2 and Bcl-X_L) genes nor in the expression of the tumor suppressor gene p53. Furthermore, the expression of IFN- γ and the cdk inhibitors p21^{Waf1} and p27^{Kip1} were analyzed. IFN- γ was detected in 37% of the sarcoidosis patients, and controls were negative ($P < 0.02$). In addition, a higher proportion of patients expressing p21^{Waf1} (58%) versus controls (12%) was found ($P < 0.005$). There was a significant correlation between the expression of IFN- γ and p21^{Waf1} ($r = 0.69$) and between p21^{Waf1} and fibronectin ($r = 0.65$). Finally, using immunohistochemistry, high p21^{Waf1} reactivity was observed inside the granuloma. We conclude that the high levels of p21^{Waf1} in sarcoidosis may explain the absence of apoptosis in the granuloma and the persistence of inflammation. *J. Leukoc. Biol.* 74: 000–000; 2003.

Key Words: apoptosis · macrophages · cdk inhibitors · p27^{Kip1} · fibronectin

INTRODUCTION

Sarcoidosis is a multisystemic, granulomatous disease of unknown etiology that involves mainly the lungs, mediastinal and peripheral lymph nodes, skin, liver, spleen, eyes, and parotid glands. Less frequent, but usually severe, manifestations also occur in the central nervous system, heart, upper respiratory tract, and bones [1]. The disease is characterized by the formation of noncaseating granulomas in the organs affected. Sarcoid granulomas typically show a compact and highly dy-

namic structure made by a central cluster of mononuclear phagocytes, and their progeny (macrophages, epithelioid, and giant cells) are generally surrounded by a rim of lymphocytes, which mostly contain CD4⁺ T cells but also a variable proportion CD8⁺ T cells and B cells [2, 3].

The prognosis of sarcoidosis is good, as in most patients, the disease is spontaneously resolved in less than 2 years or after treatment with corticosteroids and without the prolongation of inflammation or remaining fibrosis, but some cases develop chronic, progressive disease [4]. However, 2 years is too long to explain the resolution of an inflammatory response. To avoid tissue damage, in normal conditions, an inflammatory response is usually resolved in a few days. In most cases, the resolution of the inflammatory foci is done by apoptosis of activated cells [5, 6]. In the immune system, the Fas antigen and ligand are involved in the down-regulation of immune reactions by inducing apoptosis [6, 7]. The expression of the apoptosis signaling receptors, tumor necrosis factor receptor (TNFR)I and Fas, is increased on alveolar macrophages in sarcoidosis [8], and very few apoptotic cells are found in acute sarcoid granulomas, despite heavy immune cell infiltration [9–11]. By contrast, apoptotic cells are observed in other lung diseases such as interstitial pneumonia associated with collagen vascular diseases or idiopathic pulmonary fibrosis but not in sarcoidosis [9].

The absence of apoptotic death inside the sarcoid granuloma may explain the long lifespan of the cells involved in the inflammation of this tissue. In this context, this study aimed to examine the molecular mechanisms that could explain the absence of apoptosis in the granuloma despite inflammation and the expression of Fas and other death receptors.

Activation of macrophages in vitro by interferon- γ (IFN- γ) or their adhesion to extracellular matrix (ECM) decorin led to the suppression of apoptosis [12, 13]. This antiapoptotic effect is mediated by the expression of the cdk inhibitor, p21^{Waf1} [12], which is involved in the regulation of apoptosis in several other cellular models [14, 15]. As macrophages and IFN- γ play

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Received December 29, 2002; revised March 18, 2003; accepted April 2, 2003; doi: 10.1189/jlb.1202628.

crucial roles in the development of the sarcoid granuloma [16, 17], we explored whether our *in vitro* studies on macrophage apoptosis have an equivalency in an *in vivo* model of a human pathology.

Through a semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) study, we observed for the first time that p21^{Waf1} and fibronectin are significantly overexpressed in skin sarcoid granulomas and that the increase in the former is correlated with the increase in IFN- γ levels observed in sarcoidosis patients. These results indicate that the expression of p21^{Waf1} could be related to the absence of apoptosis inside the sarcoid granuloma and thus for the prolonged lifespan of the cells involved in the granuloma.

MATERIALS AND METHODS

Patients, tissue samples, and blood samples

Sarcoidosis was diagnosed in 19 individuals (eight with acute and 11 with chronic sarcoidosis) in accordance with classical criteria: a compatible clinical and radiological picture; histologic demonstration of noncaseating granulomas in one or more tissues with negative stains and cultures for mycobacteria and fungi or a positive Kveim test; and exclusion of other granulomatous diseases [1]. The diagnosis of skin sarcoidosis was confirmed by the presence of noncaseating granulomas in a cutaneous biopsy. Sarcoidosis was clinically classified as acute when the disease was in complete remission in less than 2 years and chronic when activity persisted for more than 2 years after diagnosis [4]. In all cases, a diagnostic biopsy was performed, and part of the material was used for the present study. No patient was receiving corticosteroids at the time of study. For comparison, 16 normal skin biopsies were studied. Skin biopsies that contained at least one granuloma were obtained from all sarcoid subjects and used in part for diagnostic purposes and in part for this study. Biopsy samples were frozen at -80°C . In addition, some blood samples were obtained by venopuncture. Informed consent was obtained from each patient, and the study was performed following a protocol approved by the institutional review board.

Reagents and cells

All PCR reagents were purchased from Roche Molecular Biochemicals (Indianapolis, IN). All the other products were of the highest grade available and were obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant IFN- γ was obtained from Biosource (Nivelles, Belgium). Deionized water further purified with a Millipore (Bedford, MA) Milli-Q system was used.

Jurkat (human T cell, TIB-152), Raji (human B cell, CCL-86), THP-1 (human monocyte-macrophage, TIB-202), and Detroit 551 (human skin fibroblast, CCL-110) cell lines were all obtained from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37°C in an atmosphere of 5% CO_2 .

RT-PCR

Total RNA from the skin biopsies was isolated using Trizol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol and using a polytron to homogenize the biopsy. Total RNA was diluted in RNase-free water and quantified by UV spectrophotometry. Integrity of the RNAs was checked by agarose gel electrophoresis. Only samples that showed no sign of degradation were used for RT-PCR analysis.

Samples were measured using minor modifications of a semiquantitative RT-PCR technique previously used in granulomatous samples [18]. cDNA was synthesized from 600 ng total RNA by RT using 100 pmol oligo (dT)₁₅ primer (Roche Molecular Biochemicals) and 20 U/ μl Moloney murine leukemia virus RT (Roche Molecular Biochemicals) in the presence of 10 mM each deoxynucleotide triphosphate (Roche Molecular Biochemicals), 10 mM dithiothreitol, and 5 U/ μl RNase inhibitor (RNase Guard, Roche Molecular Biochemicals) for 1 h at 42°C in a 40- μl reaction. The cDNA reaction was used

(3–4 μl) for specific amplification of reverse-transcribed gene mRNA using Taq DNA polymerase (Roche Molecular Biochemicals).

Primer sequences and PCR conditions were as follows: for Bax, 5'-GG-GAGAGCTCAAACCCCTGCCCG-3' and 5'-CCCCGTGAACCTGCCTGC-3' at 57°C , producing an amplicon of 275 bp; for Bcl-2, 5'-GGATGCGGAGATCTGGGGCG-3' and 5'-GGCACCCGGCTGAGCCAGG-3' at 57°C , producing an amplicon of 275 bp; for Bcl_{X_L}, 5'-CAGACAGCCCCGCGGTGAATGG-3' and 5'-CTGTCCGGCTGCTGCATTGTCC-3' at 57°C , producing an amplicon of 425 bp; for Bcl_{X_S}, 5'-CAGACAGCCCCGCGGTGAATGG-3' and 5'-CTGTCCGGCTGCTGCATTGTCC-3' at 57°C , producing an amplicon of 225 bp; for IFN- γ , 5'-CGAGATGACTTCGAAAAGCTGACT-3' and 5'-CCTTTTCGCTTCCCTGTTTA-3' at 57°C , producing an amplicon of 120 bp; for p21^{Waf1}, 5'-CTGGGGATGTCGTCAGAAC-3' and 5'-GACCTGCTCCCTGAGCGAGG-3' at 57°C , producing an amplicon of 350 bp; for p27^{Kip1}, 5'-GGACCGGATGAGCGCAGG-3' and 5'-TGGGGGGCCCGGGG-3' at 57°C , producing an amplicon of 250 bp; for p53, 5'-GTCGACCCCCCTCTGACTCAGG-3' and 5'-GCTGCTCAGGGGCCACCGG-3' at 63°C , producing an amplicon of 200 bp; for decorin, 5'-CCTGATGACCGGACTTCGAGC-3' and 5'-CTCCTGAAGAGTTTTGGGC-3' at 50°C , producing an amplicon of 325 bp; for Collagen IV, 5'-GGTAAAGAGGCCTCCGG-3' and 5'-CTTGCCCTTTTGTTC-3' at 57°C , producing an amplicon of 130 bp; for fibronectin, 5'-GGCAAGTTCCAGGTACAGGG-3' and 5'-CTGGCCGCTCAGGCTGTGGG-3' at 63°C , producing an amplicon of 250 bp; for glyceraldehyde 3-phosphate-dehydrogenase (GAPDH), 5'-CAACTACATGTTTACATGTTTC-3' and 5'-GCCAGTGGACTCCACGAC-3' at 57°C , producing an amplicon of 175 bp.

PCR products were separated on 1.5% agarose gels and quantified by densitometric analysis of ethidium bromide-stained gels. Negative controls, consisting of the reaction mixture without cDNA, were run simultaneously. Expression levels were compared and corrected for the expression levels of the GAPDH-retrotranscribed cDNA.

Immunohistochemistry for p21^{Waf1}

Immunohistochemistry was performed using the monoclonal mouse anti-human p21^{Waf1} antibody (BD PharMingen, Heidelberg, Germany) and a peroxidase-anti-mouse antibody (Sigma Chemical Co.) or the simultest LeucoGate (anti-CD45 and anti-CD14 labeled with rhodamine or fluorescence, respectively; Becton Dickinson, San Jose, CA). Following deparaffinization in xylene and rehydration through ethanol to water, nonspecific protein staining was blocked by rabbit serum for 30 min at room temperature. The sections were incubated overnight at 4°C with the p21^{Waf1} antibody, the combination of CD45 and CD14 antibodies, or the control isotype antibody. The sections were rinsed three times with phosphate-buffered saline 0.1% Triton X-100, incubated with the secondary antibody for 1 h at room temperature, and then washed and developed using 3'-diaminobenzidine tetrahydrochloride as a substrate (Sigma Chemical Co.). Contrast staining of the sections was performed with haematoxylin. Pictures were obtained using an Axiovert 25 microscope (Zeiss, Jena, Germany) at a 400 \times magnification.

RNA extraction and Northern blot analysis

Total cellular RNA (20 μg), extracted with the TRIZOL reagent (Life Technologies, Grand Island, NY), was separated in 1% agarose gel with 5 mM 3-(N-morpholino) propanesulfonic acid, pH 7, 0/1 M formaldehyde buffer. RNA was transferred overnight to a Hybond-XL nitrocellulose membrane (Amersham Pharmacia, Uppsala, Sweden) and fixed by UV irradiation (150 mJ). The probe, Pmh117 plasmid, kindly provided by Dr. Joan Massagué (Sloan Kettering Institute, Howard Hughes Medical Institute, New York, NY), corresponds to the p21^{Waf1} full-length cDNA cloned in pEx-lox and was labeled with ³²P- α -dCTP (ICN Pharmaceuticals, Costa Mesa, CA) using the oligolabeling kit method (Amersham Pharmacia). After incubating the membranes for 18 h at 65°C in hybridization solution (5 \times standard sodium citrate, 5 \times Denhart, 1% sodium dodecyl sulfate, and 10⁶ cpm/ml ³²P-labeled probe), they were exposed to Kodak X-AR films (Kodak Co., Rochester, NY).

Statistical analysis

As a result of difficulty in quantifying the PCR bands with small intensity in agarose gels, data were represented as nonparametric. For each sample, values were corrected with the GAPDH expression levels. We assigned a value "1" for

bands with 0–20% of the maximal expression, “2” for 21–40%, “3” for 41–60%, “4” for 61–80%, and “5” for 81–100%. Comparison of group data was done using a χ^2 test with Yates’ correction when values were 0 [19]. The nonparametric Spearman rank was used to calculate the correlation coefficient. Values of $P < 0.05$ were considered significant.

RESULTS

Although some contradictory results can be found in the literature, it is accepted that apoptotic events are reduced in sarcoid granulomas, which explains the long-lasting inflammation and the lifespan of the cells involved [9–11]. Here, we examined the molecular mechanisms that could be involved in the inhibition of apoptosis.

For this purpose, we obtained skin biopsies from 35 subjects (eight with acute and 11 with chronic sarcoidosis, and 16 controls) and obtained total RNA samples from each biopsy. Samples were subjected to semiquantitative RT-PCR analysis using oligonucleotides specific for 11 genes, and the results of the PCR are shown in **Figure 1**. The quantification of the bands was expressed as nonparametric values. Skin biopsies were used, as they are very easy to obtain, and the granulomas are identical to granulomas in any other tissue.

As no differences were found between samples from chronic and acute forms of sarcoidosis in the expression of any of the genes determined in this study, these two groups were joined to make a unique group, which was then compared with controls.

First, we examined some members of the Bcl-2 family of genes and also expression of the tumor suppressor gene p53, as these genes are involved in the regulation of apoptotic events in several cellular models (reviewed in refs. [20–22]). For this purpose, we studied the expression of proapoptotic genes such as Bax and Bcl-X_s or antiapoptotic genes such as Bcl-2 and Bcl-X_L. Because of the reduced apoptosis in sarcoid granulomas, we expected to find an increase in the expression of antiapoptotic genes or a reduction in that of proapoptotic

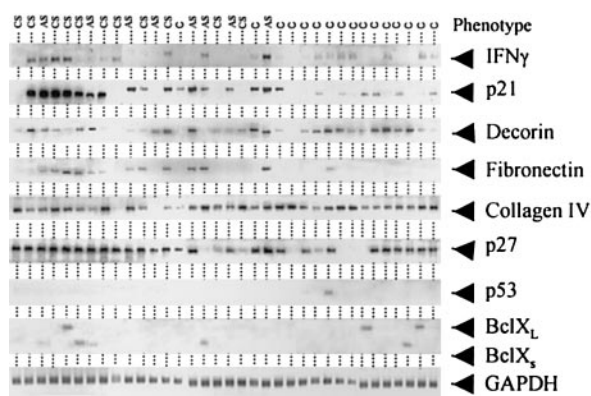


Fig. 1. Differential expression of mRNAs in biopsies of sarcoidosis. The differential expression of the indicated genes was analyzed by RT-PCR. Total RNA was isolated, and cDNA was synthesized using oligo (dT)₁₅ and RT. Specific primers for each gene are indicated in Materials and Methods. All PCR analyses were done in nonsaturating conditions (35–38 cycles in this case). The GAPDH gene was used as an internal control. The expression of Bcl2 and Bax was negative in all the cases. CS, Chronic sarcoidosis; AS, acute sarcoidosis; C, control.

TABLE 1. Expression of Apoptotic Genes in Patients with Sarcoidosis and in Controls

Gene	Control		Sarcoidosis		P^a
	Samples	%	Samples	%	
Bax	0/16 ^b	0	0/19 ^b	0	N.S.
Bcl-2	0/16	0	0/19	0	N.S.
BclX _L	1/16	6.25	2/19	10.5	N.S.
BclX _s	2/16	12.5	2/19	10.5	N.S.
p53	2/16	12.5	0/19	0	N.S.

Positive values were considered >2 . ^a Statistical analysis was performed using the χ^2 test. ^b Positive/total cases. N.S., Not significant; $P > 0.05$.

genes. However, the expression of these genes was very low in all the skin biopsies, and no differences were observed between control and sarcoid biopsies (**Table 1**), which indicates that neither Bcl-2 family members nor p53 are responsible for the low rate of apoptosis observed in skin sarcoid granulomas.

In sarcoid cells, there is an increase in Fas and TNFR expression [8]. As these two molecules are death receptors that lead to apoptosis, the mechanism that protects the granuloma from apoptosis must act by inhibiting apoptosis mediated through these receptors. IFN- γ inhibits the apoptosis induced by lipopolysaccharide/TNF- α in macrophages, and for this to occur, expression of p21^{Waf1} and inhibition of the cell cycle [12, 23] are necessary. As macrophages play a critical role in the generation of granuloma [16], and there is an increase in T helper cell type 1 (T_H1) cytokines, mainly IFN- γ [17, 24], here, we measured the expression of IFN- γ and the cdk inhibitors p21^{Waf1} and p27^{Kip1} in the biopsies.

IFN- γ expression was detected in 37% of the sarcoid biopsies, and controls were negative ($P < 0.02$; **Table 2**), confirming previous works [17, 24]. Moreover, the frequency of expression of p21^{Waf1} ($P < 0.005$) was significantly higher in the biopsies from sarcoid patients than controls (Fig. 1 and Table 2). A high frequency of patients and controls expressed p27^{Kip1} (84% and 62%, respectively). Levels of IFN- γ expression were correlated with those of p21^{Waf1} (**Fig. 2**).

In previous studies in vitro, we observed that the proteoglycan decorin present in the ECM of several tissues protects macrophages from apoptosis [13]. The mechanism of apoptosis protection by decorin involves p21^{Waf1}, in a similar way to our observations on IFN- γ [13]. Given the highly structured configuration of the sarcoid granuloma, adhesion to the ECM components could be crucial in granuloma formation [25].

TABLE 2. Expression of IFN- γ and the cdk Inhibitors p21 Waf1 and p27 Kip1 in Sarcoid and Control Skin Biopsies

Gene	Control		Sarcoidosis		P^a
	Samples	%	Samples	%	
IFN- γ	0/16 ^b	0	7/19 ^b	36.8	0.020
p21 ^{Waf1}	2/16	12.5	11/19	57.9	0.005
p27 ^{Kip1}	10/16	62.5	16/19	84.2	N.S.

Positive values were considered >2 . ^a Statistical analysis was performed using the χ^2 test. ^b Positive/total cases. N.S., Not significant; $P > 0.05$.

Moreover, decorin is a potent inhibitor of fibrillogenesis [26], and sarcoidosis is usually resolved without symptoms of fibrosis. We therefore studied whether components of the ECM are also involved in the pathogenesis of the sarcoid granuloma.

Half of the controls expressed significant levels of decorin (50%), and no differences were observed when compared with sarcoid samples (42%). Also, no differences were found in the proportion of subjects that expressed Collagen IV (87% and 79% in controls and patients, respectively; Fig. 1 and Table 3). Nevertheless, a highly significant difference was observed in the number of patients that expressed high levels of fibronectin ($P < 0.005$). Although expression of this protein was very low in the control samples, it was detected at high levels in almost 50% of the skin biopsies from sarcoidosis patients (Table 3). The frequency of subjects expressing fibronectin correlated well with genes whose expression was increased in sarcoid samples, namely p21^{Waf1} and IFN- γ (Fig. 3).

Our results demonstrate that sarcoid granulomas show an increase in expression of IFN- γ , p21^{Waf1}, and fibronectin, which could explain the long lifespan of the granuloma and therefore the pathogenesis of the disease. To establish whether the increase in the expression of these genes was a local effect inside the granuloma or a general effect, we analyzed the expression of these genes in mononuclear cells from peripheral blood of some patients. As expected, the increase in the expression of all these genes was only a local event in the sarcoid granuloma and did not affect circulating immune cells.

To confirm the possible involvement of p21^{Waf1} in the inhibition of apoptosis inside the sarcoid granuloma, we performed immunohistochemistry staining of paraffinated sections of the tissue. Although high expression of p21^{Waf1} reactivity was detected inside the granuloma (Fig. 4A), none was detected in the adjacent tissue. To control the origin of the cells inside the granuloma, we stained them with an anti-CD14 and anti-CD45, indicating that these cells are of macrophage origin (Fig. 4B). These findings confirm our RT-PCR results and the involvement of p21^{Waf1} in the pathogenesis of skin sarcoidosis.

Finally, to confirm that the immune cells with high expression levels of p21^{Waf1} inside the granuloma are macrophages, we analyzed in vitro the p21^{Waf1} expression induced by IFN- γ in several human cell types representative of the cells involved

TABLE 3. Expression of ECM Components in Sarcoid and Control Skin Biopsies

Gene	Control		Sarcoidosis		P^a
	Samples	%	Samples	%	
Decorin	8/16 ^b	50	8/19	42.1	N.S.
Fibronectin	0/16	0	9/19	47.4	0.005
Collagen IV	14/16	87.5	15/19	78.9	N.S.

Positive values were considered >2 . ^a Statistical analysis was performed using the χ^2 test. ^b Positive/total cases. N.S., Not significant; $P > 0.05$.

in granuloma. After stimulation with 100 units of IFN- γ , the expression of p21^{Waf1} was induced only in human macrophages (Fig. 5), confirming our previous results in mice [12]. This observation together with the fact that the p21^{Waf1} staining with the CD14 and CD45 markers (Fig. 4) was done in a compact structure inside the granuloma indicate that macrophages are the inflammatory cells inside the granuloma that express the high levels of p21^{Waf1}.

DISCUSSION

Macrophages play a crucial role during immune response. These cells are transported by blood to inflammatory foci in tissues where they remain until the disappearance of the inflammation [27]. Usually, inflammation is resolved by the elimination of the cells involved, such as neutrophils, activated lymphocytes, or macrophages, mainly through apoptosis [5]. However, during active inflammation, macrophages must survive inasmuch of the stressing conditions present in the inflammatory foci (including oxidative stress and growth-factor deprivation).

Although IFN- γ is present at the inflammatory foci produced by natural killer cells and T_H1-activated lymphocytes, macrophages may be protected from apoptosis [12]. Recently, a new component in the protection of macrophage apoptosis has been introduced in this scenario, which is ECM proteoglycan decorin. The attachment of this protein to macrophages protects them from apoptosis [13]. Therefore, the extracellular milieu in the inflammatory foci (cytokines, ECM proteins) determines cell survival and thus the duration of the inflammatory response. Modification of this equilibrium could result in the resolution or the persistence of inflammation. This could be the case of sarcoidosis, the pathogenesis that involves the formation of granulomas. In this context, we explored whether our results in vitro [12, 13] correlate with those obtained in vivo by using biopsies from sarcoidosis patients.

Sarcoid granulomas show a reduction in the number of apoptotic events [28, 29] in relation to acute inflammatory processes, which could affect at least 80% of the cells [5, 27]. Moreover, the presence of apoptotic events in sarcoidosis correlates with the spontaneous resolution of inflammation and the elimination of the granuloma [28, 29], which indicates that the persistence of the granuloma correlates with a reduction of apoptosis. This could explain why we did not detect differences in the apoptotic gene expression pattern between acute and

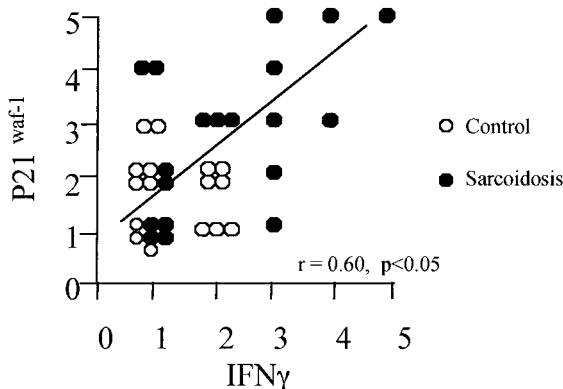


Fig. 2. Correlation between p21^{Waf1} and IFN- γ expression. The Spearman rank correlation coefficient was used to calculate the correlation coefficient between the levels of expression of IFN- γ and p21^{Waf1} in skin biopsies.

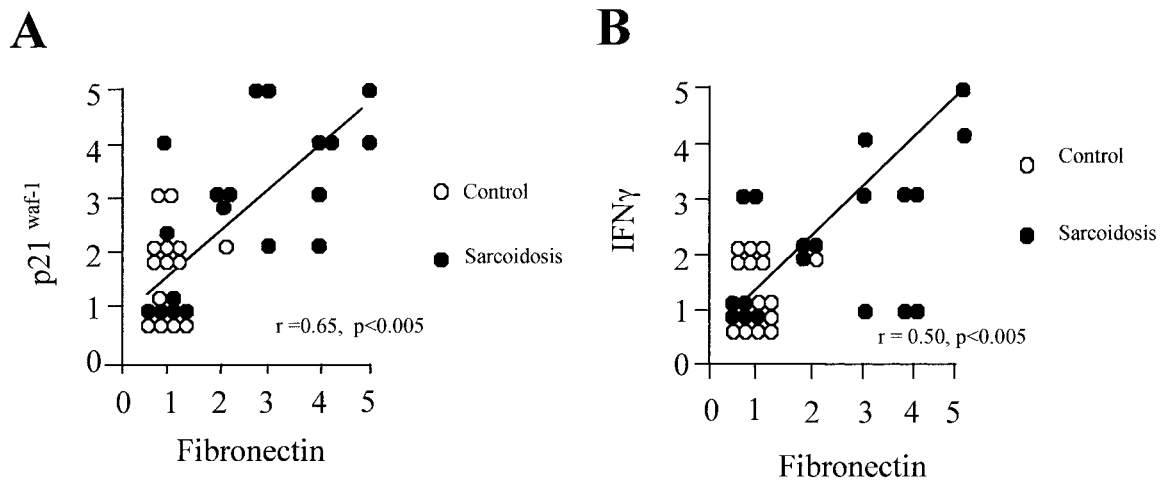


Fig. 3. Correlation between expression of fibronectin and p21^{Waf1} or IFN- γ . The Spearman rank correlation coefficient was used to calculate the correlation coefficient between the levels of expression of fibronectin and p21^{Waf1} (A) or fibronectin and IFN- γ (B) in skin biopsies.

chronic sarcoidosis, as both samples were obtained from patients with active illness.

Here, we analyzed members of the Bcl-2 family and the p53 gene, which are involved in several apoptotic pathways [20–22]. We did not observe any difference in the expression of these genes. Our results do not agree with previous reports that showed an increase in Bcl-2 gene members [11, 30] or p53 [9, 31] in sarcoid samples. This difference could be related to the tissues used. In these studies, gene expression was analyzed in

lung tissue or cells from bronco-alveolar lavages. These samples contained alveolar macrophages, which are totally differentiated cells from macrophages from blood that reach inflammatory loci in skin. In these studies, the increase in the expression of p53 or Bcl-2 members in sarcoidosis samples correlates with the presence of apoptotic cells and with the resolution of inflammation [9, 28]. This phenomenon was not observed in our biopsies, and therefore, the two situations cannot be compared.

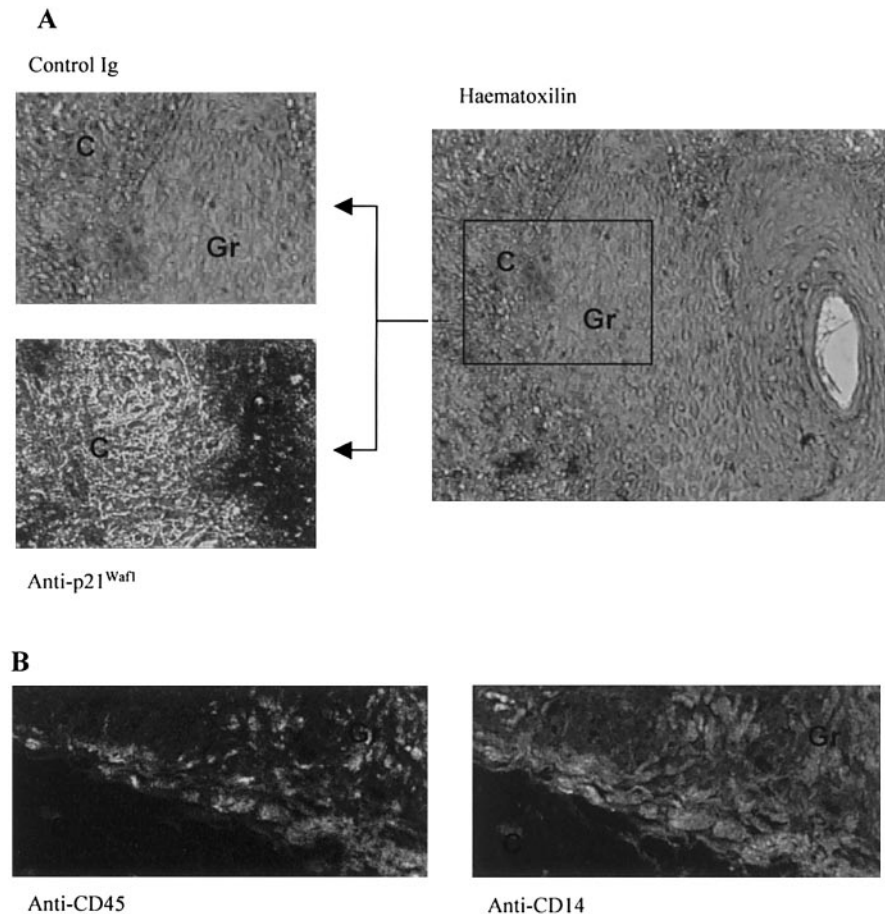


Fig. 4. Immunohistochemical analysis of p21^{Waf1} expression in a skin biopsy of a patient suffering from sarcoidosis. (A) Immunohistochemical staining was performed with a monoclonal mouse anti-human p21^{Waf1} antibody, as described in Materials and Methods. Control sections were stained with nonimmune mouse immunoglobulin (Ig) G showing a positive signal. (B) Immunohistochemical staining was performed with an anti-CD14 antibody labeled with fluorescein and an anti-CD45 antibody labeled with rhodamine, as described in Materials and Methods. All sections were contrasted by haematoxylin staining. C, Control, nongranulomatous area; Gr, granuloma area. Original magnification, 400 \times .

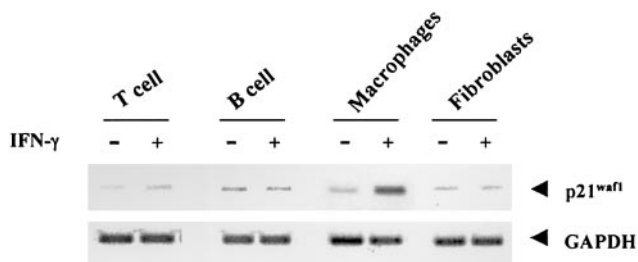


Fig. 5. In vitro induction of p21^{Waf1} expression by IFN- γ . The indicated human cell lines were incubated with IFN- γ (100 U/ml) for 24 h, and mRNA expression was measured by Northern blot (20 μ g total RNA per lane). Expression of GAPDH was used as a control for RNA loading and transfer.

In sarcoidosis, there is a prevalence of T_H1 lymphocytes, which produce increased levels of T_H1-type cytokines (our results and refs. [16, 17, 32]). However, only IFN- γ plays a crucial role in the development of granulomas. This is supported by experimental data reported by Gudmundsson and Hunninghake [33], who showed that it is not possible to induce the formation of granuloma in mice in which the IFN- γ gene has been disrupted by homologous recombination.

In our in vitro studies, we showed that the protective effect of IFN- γ is mediated by blocking cell-cycle progression and the induction of p21^{Waf1} [12]. Using RT-PCR and immunohistochemical analysis, we demonstrate that p21^{Waf1} is up-regulated in sarcoid patients and that this increase correlates with the levels of IFN- γ . Moreover, we show that the increase in p21^{Waf1} expression is a local event inside the granuloma but not a systemic effect, as circulating leukocytes from sarcoid patients did not overexpress this molecule. In the target organ (in our study, the skin), the T_H1 cytokine milieu allows for the increase of p21^{Waf1} and thus the survival of macrophages in the granuloma.

Cell survival is also regulated by adhesion to components of the ECM [34–36]. In our in vitro experiments, we found that binding macrophages to decorin inhibits apoptosis through a p21^{Waf1}-dependent pathway [13], similar to our observations with IFN- γ . Also, adhesion of macrophages to decorin increases their activity (manuscript submitted). As decorin also inhibits fibrosis [26], another characteristic of sarcoidosis, we suspected that decorin plays a key role in the formation of the granuloma. However, we did not detect an increase in the proportion of patients who express decorin.

Another component of the ECM, fibronectin, was highly expressed only in sarcoid samples and correlated with the expression of IFN- γ and p21^{Waf1}. However, macrophage activation by IFN- γ does not increase the expression of fibronectin [13], and binding to this protein does not induce the expression of p21^{Waf1} or offer protection from apoptosis [13].

Fibronectin is a component of the inflammatory reaction and may play a key role in the formation and persistence of granulomas. Blood monocytes migrate in response to fibronectin [34–36], which promotes their differentiation to tissue macrophages [37, 38]. Macrophages secrete fibronectin at the inflammatory loci and then firmly attach to the loci [13, 38], a process that is necessary for the high structuration of the granuloma. Moreover, fibronectin enhances activation of adjacent T lym-

phocytes [39, 40], which increase the production of IFN- γ . The increase in IFN- γ production is the clue in the induction of p21^{Waf1} [12], the latest responsible for reduction in the rate of apoptosis [12, 13].

Thus, together with fibronectin, the increase in IFN- γ and the induction of p21^{Waf1} could be the main agents responsible for the long lifespan of cells inside the granuloma and thus, for the long-lasting inflammation observed in sarcoidosis.

ACKNOWLEDGMENTS

This work was supported by Fondo de Investigaciones Sanitarias Grant 98/774 (to J. M.) and Ministerio de Ciencia y Tecnología Grant BMC2001-3040 (to A. C.). We thank Dr. Manel Juan from the Laboratory of Immunobiology for Research and Application to Diagnosis, Autonomous University of Barcelona, for his help in the histochemistry determinations. We thank Tanya Yates for editorial help. We also thank Dr. Alvin S. Teirstein, Mount Sinai Medical Center, NY, for advice.

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