CONCISE REPORT

Cell death by apoptosis is a feature of the rheumatoid nodule

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Objective: To examine the site and extent of apoptosis in the rheumatoid nodule and to determine whether this process make a significant contribution to the control of inflammation in the rheumatoid nodule as in other granulomas.

Methods: Nine nodules and seven synovial membranes were examined by terminal deoxynucleotidyl transferase-mediated nick end labelling (TUNEL) in situ and a subset was further examined by DNA electrophoresis. The phenotype of apoptotic cells was identified using monoclonal antibodies and immunohistology.

Results: Apoptosis occurred in all zones of the nodule and, except in one case, was not focused adjacent to the necrotic centre. Apoptosis occurred in 3.5 (4.5)% (mean (SD)) of cells in the nodule and 3.6 (3.1)% of cells in synovial membranes. Apoptosis was more common in nodule T cells (4.1 (2.9)%) than fibroblasts (1.0 (1.4)%), $p = 0.01$. Among macrophages 3.2 (4.7)% were apoptotic. Banding of DNA consistent with apoptosis was seen in two of three nodules examined.

Conclusion: Apoptosis occurs at a low level in the nodule, similar to the synovial membrane. The results suggest that two modes of cell death occur in the nodule: apoptosis, which occurs throughout the nodule; and necrosis, which is concentrated near the necrotic centre. Apoptosis was more common in infiltrating inflammatory cells than in resident fibroblasts. These results are consistent with the proposal that apoptosis of infiltrating inflammatory cells is important in controlling accumulation of cells in the rheumatoid nodule as has been established in experimental granulomas.

The rheumatoid nodule is a structured lesion with features typical of a granuloma.¹ The centre of the nodule is occupied by necrotic debris. This includes organelles and other debris of cellular origin such as mitochondria as well as a few cells with intact membranes but showing features of "degeneration". Such cell debris is presumed to result from cell necrosis.²

Investigation of experimental granulomas has shown that in the earliest stage of granuloma formation, infiltrating T cells and macrophages form into groups.³ With persistent inflammation further cells are recruited and become organised, with a central core of macrophages surrounded by lymphocytes. Development of this organised structure is dependent on cytokines. In Th1 granulomas, such as those found in *Mycobacterium tuberculosis* infection, interferon γ and tumour necrosis factor α are critical to formation of the granuloma structure.⁴ In Th2 granulomas, such as those formed in response to Schistosoma eggs, interleukin (IL)4 and IL13 are required.⁵ Current evidence shows that granulomas are dynamic lesions in which the balance between recruitment of T cells and macrophages and the apoptotic death of these cells is an important factor in the formation, persistence, and resolution of the lesions. Failure of cells to die is central to the

accumulation of cells forming a granuloma and its persistence.⁶ Factors favouring inflammatory cell survival in granulomas are thought to include the cytokines IL2 and IL15 as well as interaction with fibroblasts within the lesions.^{6,7} Conversely, resolution of granulomas can be associated with high levels of apoptosis.⁸

In this investigation we sought to establish the presence of apoptosis within subcutaneous nodules to determine if cell death by apoptosis, as well as necrosis, might contribute to the control of this characteristic rheumatoid lesion.

PATIENTS AND METHODS

Patients and samples

Rheumatoid nodules ($n=9$) were removed surgically from eight patients and synovial samples from a separate group of seven patients. The research protocol was approved by the southern regional ethical committee. All patients fulfilled the American College of Rheumatology diagnostic criteria for rheumatoid arthritis.⁹ All were rheumatoid factor positive and had radiological erosions. Patients with nodules had a mean (SD) age of 66.0 (10.8) years and disease duration of 13.2 (5.7) years. Three were taking methotrexate, three sulfasalazine, and one azathioprine. In addition two were taking low dose prednisone (7.5 and 8 mg/day). The mean age of patients providing synovial samples was 54.1 (10.2) years and disease duration 15.7 (5.8) years. Four patients were taking sulfasalazine, one aurothiomalate, and two were taking only non-steroidal anti-inflammatory drugs.

Tissue processing and immunostaining

Cryostat sections were immunostained by an indirect peroxidase method, as previously described.¹⁰ The inflammatory infiltrate in sections was characterised as monocytes/macrophages using monoclonal antibodies (mAb) specific for CD68 (clone KP1, DAKO), or T lymphocytes, using the CD3-specific mAb UCHT1, kindly provided by Dr Nancy Hogg, ICRF. Resident fibroblasts were identified using an mAb specific for prolyl-4-hydroxylase (clone 5B5, DAKO).

In situ detection of apoptotic cells

Apoptotic cells were detected with the TUNEL method (in situ cell death detection kit; Roche Molecular). For negative control specimens, TdT was excluded. Positive controls included sections in which DNA strand breakage was induced by incubating sections with a 1 mg/ml DNase I solution before the TUNEL staining. Additional positive controls included cytocentrifuged preparations of polymorphonuclear leucocytes (PMN) maintained and aged in culture for 48 hours.

Abbreviations: IL, interleukin; mAb, monoclonal antibodies; PMN, polymorphonuclear leucocytes

Figure 1 Histochemical staining of nodule sections. (A) Low power ($\times 100$) view of a nodule stained with the TUNEL method and fast red so that apoptotic nuclei are stained red. In this particular section apoptotic cells are concentrated at the edge of the necrotic zone. (B) High power ($\times 630$) view of (A). (C) Double staining by the TUNEL method with fast blue substrate resulting in blue staining of apoptotic nuclei, together with CD68 with peroxidase (brown macrophages). Full arrow indicates apoptotic nuclei without staining of the cell, and arrowhead indicates double stained cells (apoptotic macrophage). This image is taken from the outer edge of the palisade. Original magnification $\times 630$. (D) Double staining with CD3 to show apoptotic T lymphocytes (arrowhead) in an area outside the palisade including other apoptotic cells (full arrow). Original magnification $\times 630$. (E) Double staining with 5B5 to show apoptotic fibroblasts (arrowhead) in the nodule stroma including other apoptotic cells (full arrow). Original magnification $\times 630$. Note: negative control sections in which TdT was excluded were blank and showed nuclear staining with counterstain only.

Table 1 Arbitrary grading of extent of apoptosis at various sites in the nine nodules examined. Although most prominent at the edge of the necrotic centre and around peripheral vessels, on average, apoptosis occurs in all zones of the nodule

Nodule	Necrotic centre	Inner palisade	Palisade	Stroma	Perivascular zone
1	3	2	2	2	2
2	3	3	2	1	2
3	2	2	2	1	3
4	2	2	2	2	2
5	2	3	3	3	3
6	2	2	2	3	3
7	2	2	2	1	1
8	3	3	3	4	4
9	3	4	2	3	3
Mean	2.4	2.6	2.2	2.2	2.6

Immunohistochemistry in combination with TUNEL staining

For double staining, sections were first processed by the TUNEL method but were developed using fast blue substrate, resulting in blue staining of apoptotic nuclei, followed by peroxidase immunostaining (brown). Double stained cells were identified as those with a blue nucleus (apoptosis) and a brown cell body or black nucleus resulting from a combination of blue and brown with a brown cell body. Double stained sections were not counterstained.

Counting apoptotic cells

Following a validated procedure,¹¹ a single observer counted apoptotic cells as a percentage of the nucleated cells in 10 ran-

dom high power ($\times 400$) fields. In double stained sections the number of apoptotic double stained cells was expressed as a percentage of the total immunostained cells. Significant differences between counts of double stained cell populations were established using Student's *t* test.

Site of apoptosis

Sections of nodules were examined to establish whether apoptosis was confined to a particular region of the nodule. The extent of apoptosis was graded subjectively on an arbitrary scale from 0 to 4, where 0 equals no apoptosis and 4 equals the highest levels of apoptosis seen in these nodules. Given that the highest level of apoptosis seen was about 18% these grades approximate to grade 0 = 0%; grade 1 = <4%;

Figure 2: DNA electrophoresis. Equal amounts of DNA (3.5 µg) were run on 1.2% agarose gels and visualised using ethidium bromide. Lane 1, molecular weight markers; lanes 2 and 3 are PMN cultured for 48 and 24 hours; lanes 4, 5, 6 are DNA from three separate nodules; lanes 7, 8, 9 are from three separate synovial membranes. Note the banding in positive controls (PMNs), two of three nodules and one of three synovial membranes.

grade 2 = 4–8%; grade 3 = 8–12%, and grade 4 = 12–18%. Grading was made in the necrotic centre, inner palisade, palisade, stroma, and perivascular regions of the nodules.

Analysis of DNA

For individual tissue samples, DNA was extracted from 20 cryostat sections, each of 20 µm thickness. Equal amounts (3.5 µg) of DNA were subjected to electrophoresis on 1.25% agarose gels and stained with ethidium bromide. DNA extracted from cultured peripheral blood neutrophils was used as a positive control.

RESULTS

The percentage of apoptotic cells in nodules (figs 1A and B) ranged from 0.6% to 14.9%, mean (SD) 3.5 (4.5)% (table 1) and in synovial samples from 0.2% to 8.1%, mean (SD) 3.6 (3.1)%. Thus the mean number of apoptotic cells in the nodules was comparable with that found in the synovial membranes. We were interested to determine if there was an excess of apoptosis occurring in the vicinity of the necrotic centre of the nodules. Grading of apoptosis in different regions of the nodules (table 1) showed, on average, an even spread of apoptosis occurring throughout all zones of the nodule and that, with one exception, there was no concentration of apoptosis near the central necrotic zone of the nodule (figs 1A and B). Double staining (figs 1C–E) showed that 3.2 (4.7)% of macrophages, 4.1 (2.9)% of T lymphocytes, and 1.0 (1.4)% of fibroblasts were apoptotic. Significantly more T lymphocytes than fibroblasts were apoptotic ($p=0.01$, Student's t test,) but the differences between other cell types were not significant. Thus, apoptosis was most prominent in T lymphocytes and monocytes/macrophages and least in resident fibroblasts.

To confirm the presence of apoptosis using different methodology, DNA was extracted from a subset of nodules and synovial membranes and analysed by agarose gel electrophoresis (fig 2).

DISCUSSION

The potential importance of apoptosis, or the relative lack of it, has been emphasised as a driver of autoimmunity in general and of rheumatoid synovitis in particular.^{12,13} Whether this might apply to other lesions of rheumatoid arthritis such as the nodule, has not been examined. In other forms of granulomas there is evidence that apoptotic cell death is important in determining the balance between accumulation of cells in the granuloma, and resolution of the granuloma when disposal of cells exceeds recruitment. Having postulated that apoptosis might occur within the rheumatoid nodule, we sought to determine the extent to which apoptosis occurs, the location of apoptotic cells, and their type.

There was an even spread of TUNEL positive cells throughout all regions of the nodule. Concentration of such cells near the central necrotic zone was seen in only one sample. Although the possibility that cells with damaged DNA may not undergo apoptosis has been suggested—for example, in the synovial membrane,¹⁴ TUNEL positivity, together with the presence of DNA laddering, suggests that significant proportions of the TUNEL positive cells within the nodule are indeed undergoing apoptosis rather than necrosis. If this is so then cell death is occurring by two mechanisms in the rheumatoid nodule, necrosis and apoptosis. We found that the overall rate of occurrence of apoptotic cells in the nodule, at 3.5% of cells, was very similar to the 3.6% of cells affected in the synovial membrane. This figure is in the general range of results for synovial membrane found in previous investigations. We found that there was a comparable rate of apoptosis in T lymphocytes and macrophages in the nodule, but that at least for T lymphocytes, the rate was significantly higher than for fibroblasts. This suggests that apoptosis predominantly affects infiltrating inflammatory cells, rather than resident fibroblasts. This is in accord with previous observations in the synovial membrane and consistent with a lack of T lymphocyte aggregation in the nodule that might otherwise afford protection from apoptosis.^{15,16}

Overall, our results show that apoptosis does occur in the rheumatoid nodule. We consider it most likely that this takes place independently of the necrotic process focused in the centre of the nodule. The level of apoptosis is low and comparable with that occurring in the synovial membrane in rheumatoid arthritis. Furthermore, both rheumatoid lesions contain small numbers of apoptotic cells compared with those found in sarcoid granulomas, where high rates of apoptosis are associated with resolution of granulomatous inflammation.⁸ In view of the proposed importance of inflammatory cell apoptosis in controlling synovial inflammation in rheumatoid arthritis, and in determining the growth and resolution of granulomas such as the rheumatoid nodule, it would be relevant to investigate pathways controlling apoptosis in the rheumatoid nodule and the effect on this of drugs such as methotrexate.

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