Cell death by apoptosis is a feature of the rheumatoid nodule

J Highton, P A Hessian, A Kean, M Chin

**Objective:** To examine the site and extent of apoptosis in the rheumatoid nodule and to determine whether this process makes 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.

**Tissue processing and immunostaining**

Cryostat sections were immunostained by an indirect peroxidase method, as previously described.19 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.
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, a single observer counted apoptotic cells as a percentage of the nucleated cells in 10 random high power (×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%;
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, 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.13 14

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.4 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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REFERENCES

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