Papers

Extent of apoptosis in ovarian serous carcinoma: relation to mitotic and proliferative indices, p53 expression, and survival

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Abstract

***Aims*—To assess the extent of apoptosis in ovarian serous carcinoma and to examine possible relations between apoptosis, cell proliferation, p53 overexpression, and patient survival.**

***Methods*—Apoptotic and mitotic indices were obtained by examining haematoxylin and eosin stained sections from 30 patients with ovarian serous carcinoma. Apoptosis was also evaluated semiquantitatively by in situ end labelling of fragmented DNA. Expression of p53 and determination of Ki-67 labelling indices were based on immunohistochemical staining. Clinical details were obtained from patients' clinical records. For statistical analysis, Fisher's exact test, parametric (Pearson) linear correlations, and the Kaplan-Meier method were used.**

***Results*—The mean apoptotic index was 1.3% (range 0.02–3.9%), the mean mitotic index was 0.4% (range 0.02–1.1%), and the mean Ki-67 labelling index was 16% (range 4–32%). There were significant correlations between the apoptotic and mitotic indices ($p < 0.0205$) and between the mitotic and Ki-67 labelling indices ($p < 0.024$). There was a significant correlation between a high apoptotic index and poor prognosis ($p < 0.02$). p53 was overexpressed in 16 cases but the extent of apoptosis and outcome were both independent of p53 status.**

***Conclusions*—These results suggest that regulation of apoptosis is an integral component of tumour cell kinetics in ovarian serous carcinoma, and that increased apoptosis is indicative of aggressive tumour growth. p53 expression did not correlate with altered apoptosis, but the possibility of an attenuated apoptotic response to subsequent DNA damage by anticancer agents is not excluded.**

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Apoptosis is the highly conserved, genetically mediated process of selective cell deletion that occurs in embryogenesis, normal tissue homeostasis, and in many human diseases.[1 2] Apoptosis is fundamental in tumour cell kinetics,[1] and susceptibility to apoptosis in untreated malignant tumours varies with tumour type.[3] The precise mechanisms that regulate tumour cell apoptosis in vivo are not understood fully but there are numerous endogenous and exogenous initiating factors including growth factor deprivation, viral infection, irradiation, and cytotoxic chemotherapy.[1 4] In addition, apoptosis is subject to modulation by a large number of proto-oncogenes and tumour suppressor genes including c-myc, bcl-2, and p53.[1 5]

Ovarian cancer is the leading cause of death in industrial countries among women with carcinomas of reproductive organs.[6] Most patients present with advanced disease, management of which consists of debulking surgery followed by chemotherapy.[6–8] The principal prognostic factors are patient age, stage of disease, residual disease after surgical resection, and histological grade.[8–11] Multiple genetic changes, including allele loss[12–14] and oncogene and tumour suppressor gene abnormalities[15–22] have been described in ovarian carcinoma, particularly in serous carcinoma. p53 gene mutations and abnormal stabilisation of the p53 protein have been reported to correlate with late clinical stage, high grade, and presence of residual disease in serous carcinoma.[19–22] Recently, it has been suggested that p53 mutations are associated with reduced susceptibility to chemotherapy induced apoptosis.[22]

We examined the extent of apoptosis in ovarian serous carcinoma in relation to mitotic and proliferative indices, abnormal p53 expression, and survival.

Materials and methods

All cases diagnosed as ovarian serous carcinoma during 1990–95 were retrieved from the files of the department of histopathology, St James's Hospital, Dublin. Material consisted of formalin fixed, paraffin wax embedded sections of biopsy or resection specimens. Following review and the inclusion of additional material from the Coombe Lying-In Hospital, Dublin,

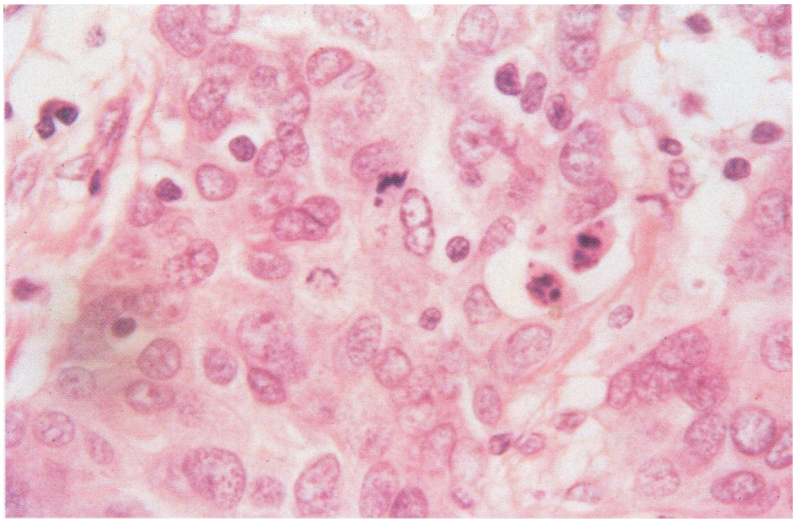

Figure 1 Apoptotic tumour cells (right of centre) in ovarian serous carcinoma.

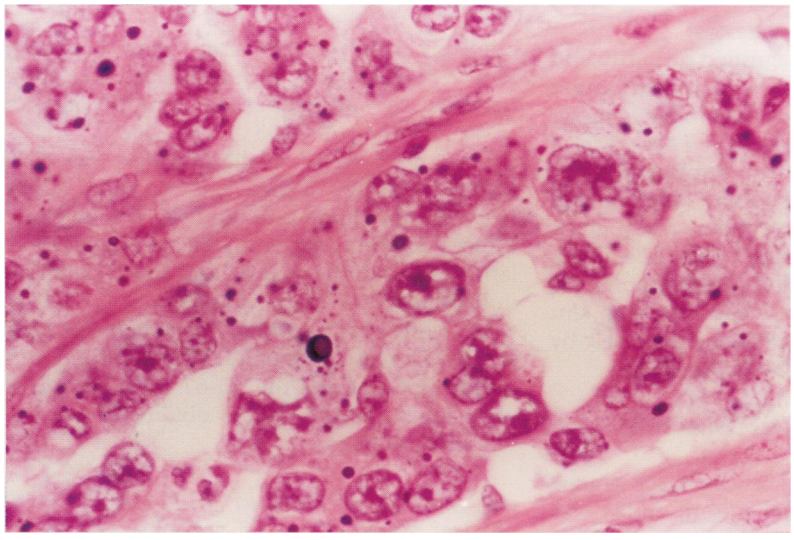

Figure 2 Multiple intracytoplasmic basophilic inclusions simulating numerous apoptotic bodies seen in one case.

30 cases of ovarian serous carcinoma formed the basis of this study. Two specimens were available from two patients, each of whom had biopsies taken before and after chemotherapy. All other specimens were from untreated patients. Each tumour was assigned a pathological grade.

One or two haematoxylin and eosin stained sections were selected for evaluation of apoptosis, mitoses, and immunostaining. The apoptotic and mitotic indices were assessed as described previously.[3] Briefly, the number of apoptotic tumour cells and mitoses in 10 high power (×40) fields (defined by a 1 mm index 100 square grid) was expressed as a percentage of the total number of tumour cells in these 10 fields. The edges of necrotic zones were avoided as were areas containing a high proportion of stromal cells.

Immunohistochemical staining for p53 and Ki-67 was performed using microwave induced antigen retrieval and a standard avidin-biotin complex method, using appropriate controls.[23] Sections were incubated in monoclonal mouse anti-p53-DO7 (Novacastra, Newcastle upon Tyne, UK; 1/50 dilution), overnight at 4°C, and monoclonal rabbit anti-Ki-67 (Dako, High Wycombe, Bucks, UK; 1/80 dilution), at room temperature for one hour. The colour reaction product was obtained with diaminobenzidine (seven minutes) and a light haematoxylin counterstain. The p53 immunoreactivity was graded initially on a scale of 0–3 (0, all tumour cells negative; 1, < 1% (weakly) positive tumour cells; 2, multifocal aggregates of uniformly staining tumour cells; 3, diffuse positive staining throughout the tumour). Subsequently, grades 0 and 1 were considered to be p53 negative, and tumours graded as 2 or 3 were considered to be p53 positive.[23] Cell proliferation was assessed by the Ki-67 labelling index, the percentage of Ki-67 positive cells within the grid boundaries of 10 non-selected ×40 objective fields.

In situ end labelling was performed using an apoptosis detection kit (Apoptag; Oncor Inc, Gaithersburg, Missouri, USA). The principle of in situ end labelling is that terminal deoxynucleotidyl transferase (TdT) catalyses the binding of digoxygenin-dUTP to the 3'-OH ends of fragmented DNA in apoptotic cells.[24] Using antidigoxygenin peroxidase, and a methyl green counterstain, a brown colour reaction product identifies apoptotic nuclei. In situ end labelling was assessed semiquantitatively, using a five tiered scale.[24]

Clinical details and follow up were obtained from hospital charts and patients' general practitioners. Survival was calculated from the date of surgery until death or the last date seen alive by the patient's doctor. From raw data entered in 2×2 contingency tables or in columns (Instat, Graphpad Software, San Diego, California, USA), p values were obtained using Fischer's exact test and parametric (Pearson) linear correlations. Survival was investigated as a function of the apoptotic and mitotic indices and p53 using Cox's proportionate hazard regression, allowing for censoring. Survival curves were displayed by means of a Kaplan-Meier plot. The group difference was tested by the log rank test.

Results

CLINICAL DATA, STAGE, AND GRADE

The mean patient age was 57 years (range 27–81 years). There were 3 stage 1, 1 stage 2, 20 stage 3, and 6 stage 4 carcinomas. Pathological grades were: grade 1 (3), grade 2 (9), and grade 3 (18). Survival data was available for 29 patients: 12 were alive and 17 were dead. Follow up intervals ranged from one to 62 months (mean 18.2 months).

ASSESSMENT OF APOPTOSIS (GENERAL)

Apoptotic tumour cells and apoptotic bodies were scattered among viable tumour cells (fig 1). The degree of apoptotic and mitotic activity showed considerable variation from field to field in grade 2 and grade 3 tumours, but this was accounted for by the assessment of 10 random high power fields. The number of tumour cells in a grid area varied with cell size and architectural pattern, and the mean number of tumour cells counted per case was 5524 (range 2190–10 662). An abundance of intracytoplasmic basophilic granules in one case simulated

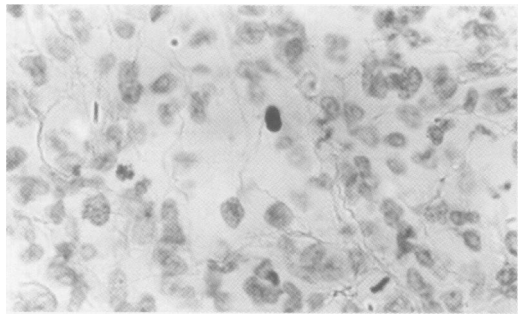

Figure 3 One apoptotic tumour cell (centre) is highlighted by the in situ end labelling technique.

very extensive apoptosis (fig 2). However, these basophilic inclusions had focal calcific laminations like miniature psammoma bodies using the von Kossa stain and a distinctive ultrastructural appearance which differed from that of apoptotic bodies.

APOPTOSIS (IN SITU END LABELLING)

Apoptotic tumour cells identified by in situ end labelling were scattered in a similar distribution but were better highlighted than in haematoxylin and eosin sections (fig 3). There was a general correlation between semiquantitative assessments made on in situ end labelling and haematoxylin and eosin sections. Frequently, however, apoptotic bodies were unstained, although morphologically recognisable, as noted previously.[24] In a few specimens, in situ end labelling staining was affected adversely by suboptimal fixation and tissue processing.

APOPTOTIC, MITOTIC, AND PROLIFERATIVE INDICES

The mean apoptotic index was 1.3% (range 0.02–3.9%) and the mean mitotic index was 0.4% (range 0.02–1.1%). Higher apoptotic indices were associated with grade 2 and grade 3 tumours: the mean apoptotic index in each grade was 0.6% (grade 1), 1.1% (grade 2), and 2.04% (grade 3). This was not statistically significant largely because of the variability of the apoptotic index in higher grade tumours. Moreover, there was no correlation between the mitotic index and grade. The lack of correlation between tumour grade and the apoptotic and mitotic indices might be due, in part, to the relatively small number of cases analysed. However, there was a close linear correlation between the apoptotic and mitotic indices ($r = 0.4210$; $p < 0.0205$).

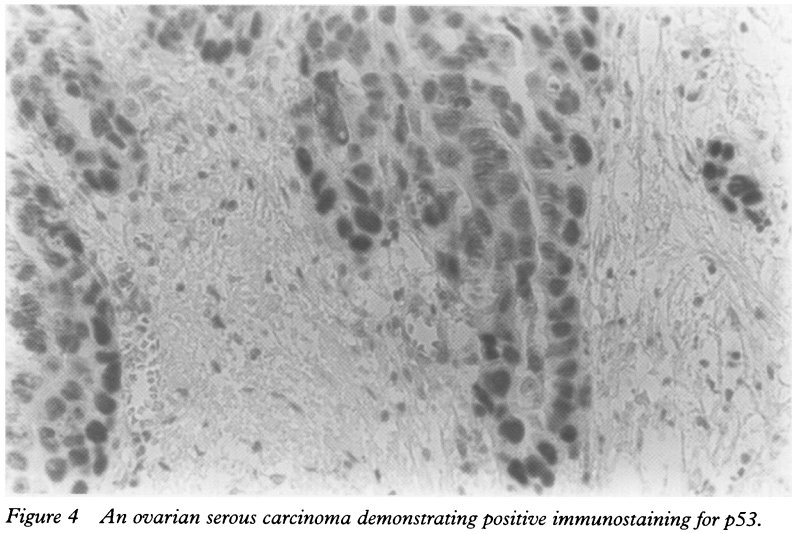

Figure 4 An ovarian serous carcinoma demonstrating positive immunostaining for p53.

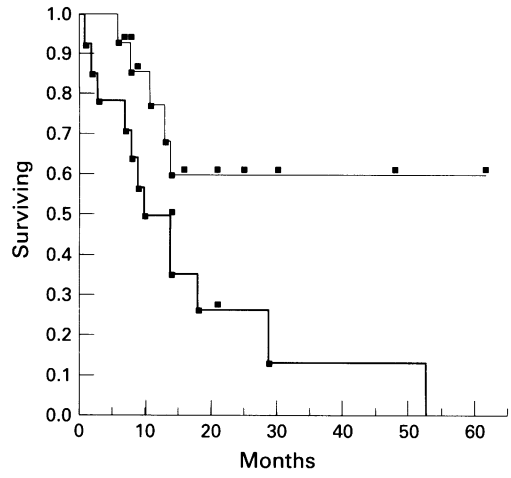

Figure 5 Kaplan-Meier survival plot: survival associated with an apoptotic index < 1.3% (upper curve) is significantly better than that associated with an apoptotic index ≥ 1.3% (lower curve) ($p < 0.02$).

The mean Ki-67 labelling index was 16% (range 4–32%). There was no correlation between the apoptotic and Ki-67 labelling indices, but there was a significant linear correlation between the mitotic and Ki-67 labelling indices ($r = 0.4114$; $p < 0.0239$).

In both cases where postchemotherapy specimens were examined, the apoptotic and mitotic indices and the Ki-67 labelling index were similar to the indices obtained from the original specimens.

IMMUNOHISTOCHEMICAL STAINING FOR P53

Nuclear staining for p53 was observed in 16 cases. Staining intensity was variable and was often maximal in peripheral tumour cells (fig 4). No cytoplasmic immunoreactivity was seen. Apoptotic tumour cells were distributed in a similar pattern in p53 positive and p53 negative tumours. There was no correlation between p53 overexpression or grade of p53 immunoreactivity and the apoptotic, mitotic or Ki-67 labelling indices.

CLINICOPATHOLOGICAL CORRELATIONS

The extent of apoptosis correlated with survival time (median survival, 14 months). A cut off point of apoptotic index < 1.3% was selected subsequently for display of Kaplan-Meier survival curves (fig 5). The difference in survival between the low and high apoptotic index groups (log rank test, χ^2) was statistically significant ($p < 0.02$). In contrast, there was no correlation between the mitotic index, the Ki-67 labelling index, or p53 overexpression, and survival.

Discussion

Apoptosis in human tumours may be inconspicuous or extensive, depending on tumour type.[3 23 25] We found a moderate amount of apoptosis in ovarian serous carcinoma: apoptotic indices (mean 1.3%) were comparable to those of adenocarcinoma of the lung and

certain other common cancers.[3 23] As reported previously,[3 26] apoptosis was identified readily and measured by light microscopy using standard haematoxylin and eosin sections. Early apoptotic nuclear changes not identified reliably by routine light microscopy were detected by in situ end labelling, a technique that identifies fragmented apoptotic cell DNA.[24 27] In general, our semiquantitative apoptosis assessments based on haematoxylin and eosin and in situ end labelling sections were comparable and the apoptotic index range was similar to that reported by Diebold *et al*,[28] who used an end labelling technique only. The limitations of end labelling techniques have been discussed fully elsewhere.[24 27 29 30] Haematoxylin and eosin sections were required for the purpose of this study, in order to measure apoptosis and mitoses in the same tumour fields.

Our results indicate that there are close links between apoptosis and cell proliferation in ovarian serous carcinoma. We found a significant linear correlation between the apoptotic and mitotic indices, as reported recently in certain other tumour types.[23 31-35] The Ki-67 labelling index, a measure of the percentage of proliferating cells, correlated significantly with the mitotic index. Furthermore, we observed that a high apoptotic index correlated very significantly with a poor prognosis in ovarian serous carcinoma. These findings suggest strongly that: regulation of apoptosis is an integral component of tumour cell kinetics, as in normal tissue homeostasis; and in certain tumour types, increased apoptosis is at least as significant as increased mitotic activity in reflecting aggressive tumour growth. Tumour growth is determined by the percentage of proliferating cells, cell cycle time, and the amount of cell loss.[36] It has been suggested that apoptosis is in some way "defective" or inhibited in cancer.[26 36-38] Data reported herein and elsewhere do not support the universal applicability of this concept: untreated carcinomas appear to display more, not less, apoptosis than normal tissues.[23 33-35] However, we cannot exclude the possibility that in tumours showing increasing genomic instability affecting specific cell cycle regulatory genes, the tightly controlled process of apoptosis might become deregulated.

The p53 phosphoprotein is a transcription factor that controls numerous cellular genes, including those involved in regulation of the cell cycle.[39] The p53 protein also induces growth arrest or apoptosis following DNA damage. In this study of ovarian serous carcinoma, the extent and distribution of apoptosis was independent of p53 overexpression, and p53 was not of prognostic significance. Ovarian carcinomas with p53 mutations are less susceptible to chemotherapy induced apoptosis, possibly because of an inability to transactivate the apoptosis inducing gene bax.[40] However, the role of p53 in regulating the extent of tumour cell apoptosis in vivo is not clear.[41] Different p53 mutants have different biological effects.[39 42] Even if all p53 mutant proteins were associated with diminished apoptosis inducing functions, p53 might not be required for fine tuning apoptosis in all untreated human tumours. With regard to the present study, it is accepted that immunostaining for p53 is not as specific as polymerase chain reaction–single strand conformational polymorphism analysis as a screening technique for p53 mutations, although there is good concordance.[19] We cannot exclude the possibility that abnormal stabilisation of wild-type p53 protein in a minority of our p53 positive cases may have been associated with a higher apoptotic index. However, we are satisfied that even if this were so, the scatter of apoptotic indices in this series of ovarian serous carcinoma indicates that p53 abnormalities were not associated with alterations in the extent of apoptosis. In support of this, recent work on non-small cell lung carcinoma and leiomyosarcoma suggests that mutations in the "hot spot region" of p53 are not associated with attenuated apoptosis (O'Neill, Staunton, McMahon, Gaffney, unpublished data, 1997). The likely significance of p53 mutations in spontaneous human tumours is that the apoptosis response to subsequent DNA damage due to chemotherapy or radiation therapy might be reduced, as the experimental evidence suggests.

Does susceptibility to apoptosis in vivo predict an apoptotic response to radiation therapy or chemotherapy? Levine *et al*[31] reported that a low apoptotic index in cervical carcinoma correlated with radiosensitivity and a favourable prognosis. However, there is no in vivo evidence that the apoptotic index might be useful for predicting clinical responses to chemotherapy (or chemoresistance) in ovarian serous carcinoma or other malignancies. Leukaemias, germ cell tumours, and small cell undifferentiated carcinomas—all chemosensitive malignancies—have low, intermediate, and high apoptotic indices, respectively.[3] Furthermore, anticancer agents induce apoptosis by several different mechanisms,[43 44] and mechanisms of chemoresistance, including those associated with bcl-2 overexpression or p53 mutation,[40 45] are further complicated by intratumour heterogeneity. We consider it likely that susceptibility to apoptosis and chemosensitivity in tumours represent independent variables, but this clinically relevant topic merits more detailed investigation.

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