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Letters to the Editor

Fibroblast growth in the soft agar clonogenic assay for cervix cancer radiosensitivity

Sir,

Recently, West and colleagues published a paper in this journal summing up their work on radiosensitivity testing in 128 cervix cancer patients, with a follow-up time from 2 to 5 years (West et al, 1997). The purpose of that study was to test the hypothesis that tumour cell in vitro radiosensitivity measured before treatment predicts clinical outcome of the individual patients after curative radiotherapy alone (Davidson et al, 1990). Tumour biopsies were obtained before treatment, and cellular in vitro radiosensitivity was assessed, using the modified Courtenay–Mills soft agar clonogenic assay, by measuring the fraction of cells surviving after a radiation dose of 2 Gy (SF_2). The study concluded that SF_2 was a significant prognostic parameter for overall survival, local control and metastasis-free survival, and that this was independent of disease stage, tumour grade, patient age, colony-forming efficiency and tumour diameter. The biological explanation for this finding is still not clear-cut, as we now demonstrate, that when culturing biopsies from carcinoma of the uterine cervix, both stromal fibroblasts as well as tumour cells can be grown.

Our approach for culturing biopsies from carcinoma of the uterine cervix involves removing the soft agar, and all the colonies are collected on preparation slides for identification of the origin of those colonies. For immunocytochemistry, the monoclonal antibodies anti-cytokeratin (AE1–3, Biogenex), reacting with epithelial cells, and anti-vimentin (3B4, Dako), reacting with fibroblasts, are used (Stausbøl-Grøn et al, 1995; 1998). Twelve carcinomas of the uterine cervix (nine squamous cell carcinomas, two adenocarcinomas, one adenosquamous carcinoma) met the criteria for successful growth, with more than ten colonies in the unirradiated tubes. Plating efficiency, irrespective of cell type ranged from 0.004% to 0.297% with a median of 0.021%, concordant with the results reported by West et al (1997). The minority of the colonies in the unirradiated cultures of most cervix carcinoma biopsies was tumour marker positive, ranging from 0% to 93%, with a median of 26% (Figure 1). In parallel, the unirradiated tubes contained 6–100% fibroblast marker-positive colonies, with a median of 80%. The sum ranged from 80% to 125%. Cellular in vitro radiosensitivities of tumour cells (tumour cell SF_2), fibroblasts (fibroblast SF_2) and an overall estimate (overall SF_2) were determined. In ten patients, overall SF_2 ranged from 0.31 to 0.81, with a median of 0.57. Tumour cell SF_2 and fibroblast SF_2 had median values of 0.53 (range 0.26–0.67) and 0.55 (range 0.28–1.00) respectively.

Previous studies on other tumour types support the finding that primary tumour biopsies are a source of fibroblast colonies when grown in the modified Courtenay–Mills soft agar clonogenic assay (Lawton et al, 1994; Stausbøl-Grøn et al, 1995). In head and neck carcinomas, the majority of the colonies obtained in the unirradiated tubes originated from fibroblasts, and the overall SF_2 was statistically significantly correlated to an independent measure of fibroblast SF_2 (Stausbøl-Grøn et al, 1995). Thus, taken together, it may be likely that the radiosensitivity of stromal fibroblasts, dominating the pretreatment measure of overall SF_2, predicts clinical outcome after curative radiotherapy in cervix cancer. However, this suggestion needs to be tested in another setting, as to our knowledge, no other study has yet found a significant correlation between the cellular in

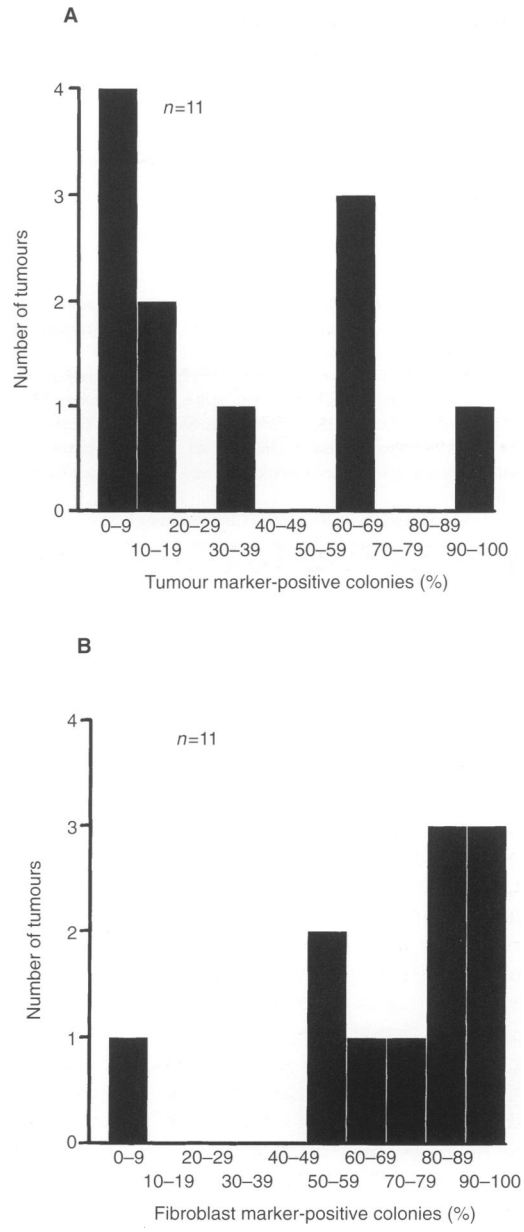

Figure 1 (**A**) The percentage of tumour cell colonies out of the total number of colonies in the unirradiated tubes. (**B**) The percentage of fibroblast colonies out of all colonies in the unirradiated tubes. One patient biopsy was omitted from the figure because the patient material was insufficient for both immunostainings

vitro radiosensitivity of tumour specimens (Brock et al, 1990; Eschwege et al, 1997) or derived cell lines (Allanunis-Turner et al, 1992; Ramsay et al, 1992; Taghian et al, 1993), measured by the overall SF_2, and the clinical outcome of individual patients after curative radiotherapy in any tumour type.

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Reply to the letter from B Stausbøl-Grøn et al

Sir,

The above letter reports the predominant growth of fibroblasts rather than tumour cells in soft agar cultures of cervix carcinomas. It then suggests that results generated by my co-workers and I (West et al, 1997) show that 'it may be likely that' fibroblast radiosensitivity predicts clinical outcome after radiotherapy in cervix cancer (Stausbøl-Grøn et al, 1998). There are four reasons why I believe they cannot translate their experience to our results.

First, there is the issue of antibody cross-specificity. This problem is most clearly demonstrated from a study on human lung tumours cultured in soft agar (Lawton et al, 1995). In the worst case, three of four colonies examined showed positive staining with cytokeratin markers and eight of eight colonies showed positive staining with a fibroblast marker (5B5). That is, for the same tumour, 75% of the colonies grown were tumour marker positive and 100% were fibroblast marker positive. The 5B5 monoclonal antibody stains prolyl 4-hydroxylase, which is involved in collagen synthesis and has been reported to be specific for fibroblasts (Esterre et al, 1992). However, both human endothelial (Schwachula et al, 1994) and macrophage (Labat et al, 1991) cells have been shown to express 5B5. Although, to my knowledge, it has not been studied in tumours, it is possible that some will express 5B5. The cervix tumour work of Stausbøl-Grøn and co-workers (1998) used the anti-vimentin monoclonal antibody 3B4 as a marker for fibroblast cells. Unfortunately, vimentin expression is not fibroblast specific. Vimentin expression in tumour cells may be a feature of dedifferentiation, and it has been reported for a variety of tumour types: breast tumours (Gould et al, 1990), sarcomas (Gerharz et al, 1990), carcinomas of the vulva (Weikel et al, 1996), lung carcinomas (Blobel et al, 1984) and endometrial carcinomas (Mobus et al, 1993). In addition, in a series of four cervix carcinoma lines, two squamous cell carcinomas were shown to be positive for vimentin expression (Kelland et al, 1987). To complicate the situation further, tumours can not only express vimentin but they can also lose keratin expression during cell division (Lane et al, 1982) and culture (Mackay et al, 1990). In support of this, rapidly proliferating tumours have been shown to have a lower proportion of cytokeratin-positive tumour cells (Wingren et al, 1995).

In our own work, we have looked at low-molecular-weight cytokeratin expression in some cervix tumours (Davidson et al, 1992). Nine tumours were examined using CAM5.2 (recognizing cytokeratins 8, 18 and 19) and CK1 (recognizing cytokeratins 6 and 18) antibodies, and positive staining of colonies was seen. Although the majority was positive using both stains, two tumours were CAM5.2 positive and CK1 negative, showing the importance of using multiple cytokeratin markers.

Secondly, there is a difference in the method used by the Aarhus and Manchester groups. In culturing human tumours, we use an enzyme cocktail followed by a half-hour disaggregation in trypsin (omitted by the Aarhus group), which is used at a fivefold higher concentration than used for the routine subculture of monolayer cells. Trypsin is known to be toxic to fibroblasts and must be used at a low concentration for a short period of time. Indeed, selective trypsinization is used in establishing epithelial cultures to prevent overgrowth by fibroblasts (e.g. Schumann et al, 1988). In addition, we have quantified the cell types present in cell suspensions prepared using enzymes from cervix tumours (Davidson et al, 1992). The predominant cell type present is tumour (mean 45%) followed by granulocyte (mean 24%), lymphocyte (mean 16%) and macrophage (mean 15%). Fibroblast-like cells are seen rarely.