

# Clinical studies of apoptosis and proliferation in breast cancer

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## Abstract

The interaction between cell death and cell proliferation determines the growth dynamics of all tissues. Studies are described here which relate the changes in proliferation and apoptosis that occur in human breast cancer during medical therapeutic manoeuvres. Xenograft studies strongly support the involvement of increased apoptosis as well as decreased proliferation after oestrogen withdrawal, and limited studies in clinical samples confirm the involvement of both processes. Cytotoxic chemotherapy induces increases in apoptosis within 24 h of starting treatment. However, after 3 months therapy the residual cell population shows apoptotic and proliferation indices much below pretreatment levels. Further molecular studies of this "dormant" population are important to characterise the mechanism of their resistance to drug therapy. The early changes in proliferation and apoptosis may provide useful intermediate response indices.

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## Introduction

Cell death, in the form of apoptosis, and cell proliferation are the key determinants of the growth of all normal tissues. The data presented here indicate that these two factors also determine the growth dynamics of breast carcinomas, including their response to a variety of therapeutic manoeuvres.

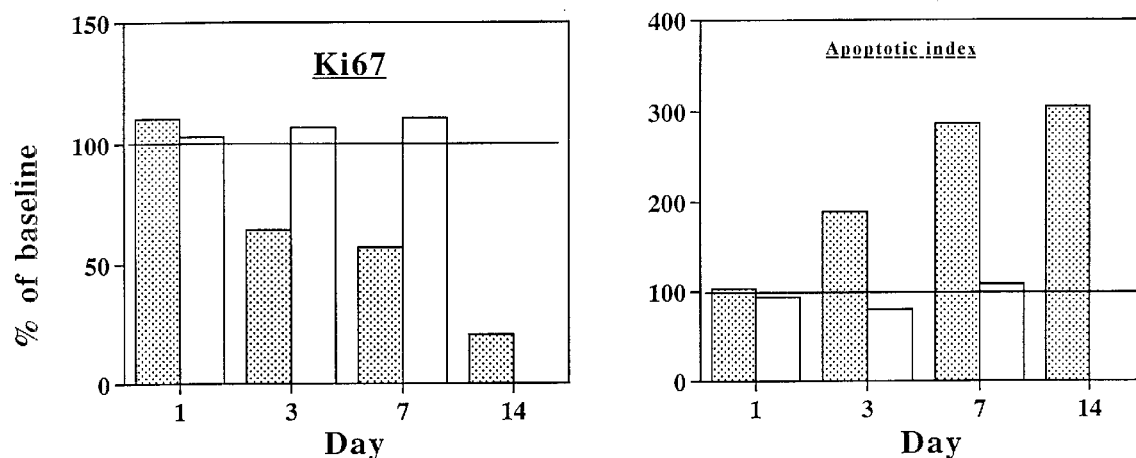
The characterisation of treatment-induced changes in proliferation and apoptosis in breast cancer is essential to our understanding of the molecular mechanisms that determine a tumour's response or resistance to therapy. In addition, early changes in these two parameters may be of use in predicting whether individual patients may be responsive to a particular therapeutic option; if so, these measures may form clinically utilizable intermediate end-points of treatment response or benefit. The study of these changes in human breast carcinomas *in vivo* is best conducted in the setting of primary medical therapy or neoadjuvant therapy; this allows serial small biopsies to be taken during treatment of primary breast carcinomas, the clinical response of which can also be observed over a period of months.

There is much discussion about the involvement of necrotic cell death in this relationship, but the areas of

necrosis that occur in breast carcinomas do not appear to be the 'driving force' behind regression of breast tumours. Indeed, the accumulation of necrotic material implies that it is not cleared from the breast carcinoma and that it cannot account for the shrinkage of the tumour during regression.

## Method validation

To allow valid interpretation of changes in proliferation and apoptosis, which we generally measure by Ki67 staining using the MIB 1 antibody (Ellis *et al.* 1998) and the TUNEL staining procedure on sections (Mainwaring *et al.* 1998) respectively, it is essential to know the overall variability of the approaches. For this reason, we examined values for each of these parameters in pairs of core-cut biopsies (14-gauge) which were derived from the same primary breast carcinoma without an interval of time or intervening treatment (Ellis *et al.* 1998). The mean±s.d. of the difference was 33±16% for Ki67 and 38±22% for apoptosis, respectively. In essence, these analyses indicate that, for an individual tumour, respective values must vary by at least 50% between two measurements for this difference to be considered statistically significant.



**Figure 1** Change in Ki67 and apoptotic index in MCF7 xenografts in athymic mice 1, 3, 7 and 14 days after removal of oestradiol pellets (stippled bars) or sham operation (open bars), expressed as a percentage of the values in tumours before pellet removal (baseline).

### Oestrogen deprivation in MCF7 xenografts

In addition to our clinical studies of mechanisms of tumour remission in response to endocrine agents, we have conducted a series of studies on xenografts, which allow more frequent measurements than in the clinic (Detre *et al.* 1999). Changes of greater degree may be expected in these studies than from clinical material, as most xenografts show quicker growth and regression than do their human equivalents.

Hormonal treatment of breast cancer has generally been considered to be cytostatic, implying that there is no induction of cell death. Over recent years, however, there have been a number of publications in which increased cell death has been recorded after endocrine manoeuvres in animal model systems (Bardon *et al.* 1987, Kyprianou *et al.* 1991, Warri *et al.* 1993, Cameron *et al.* 1997), although this has not been a universal observation (Osborne *et al.* 1995). In one of the positive studies, Kyprianou *et al.* (1991) demonstrated that oestrogen deprivation of the MCF7 human breast cancer cell line grown as a xenograft led to a two- to threefold increase in apoptosis within 2 days of the start of treatment. In a series of similar experiments, we have confirmed these data (Fig. 1) and also demonstrated that the increase in apoptosis and decrease in proliferation persists for at least 16 weeks (Boeddinghaus *et al.* 1999), implying that the very small residual tumours at that time remain in negative growth. These studies have also demonstrated that, even to achieve stabilisation of disease, as was seen in our model with tamoxifen, marked changes in proliferation and apoptosis were required: a reduction in the proliferation index by half and a two- to threefold increase in the apoptotic index were associated with growth changes that would have

been clinically termed stable disease. Although it is clear that such marked effects might not be necessary to achieve remission in a clinical setting in which the breast carcinoma may not be growing so rapidly, the principle remains: increased apoptosis or decreased proliferation should result in reduced growth rate, but such changes may not be sufficient to cause a clinical regression. Most of these changes in apoptosis and proliferation are similar to those which were observed by Cameron *et al.* (1997) with tamoxifen treatment of the ZR-75 cell line.

The xenograft model also allows the investigation of potential molecular markers of proliferation and apoptosis. For example, in our study we have demonstrated that, whereas Bax expression is largely unchanged, Bcl-2 levels are reduced, leading to an overall increase in the Bax/Bcl-2 ratio, consistent with these factors being involved in the increase in apoptosis and confirming the relationship seen by others *in vitro* (Teixeira *et al.* 1995). However, we have not found decreases in Bcl-2 expression during the early stages of anti-oestrogen treatment of breast cancer patients (Mainwaring *et al.* 1997). In addition, it is possible to observe changes in key regulators of the cell cycle. In our study, levels of cyclin D1 were somewhat variable but, overall, there was a marked reduction after oestradiol deprivation, with the changes starting within 1 day. Similarly, p27<sup>Kip1</sup>, which is a cyclin-dependent kinase inhibitor, increased markedly, with significant changes after 1 day and maximal changes after 3 days. It is notable that these changes occurred before any measurable change in Ki67, indicating that, by the measurement of these cell cycle or apoptotic controls, one may be able to predict changes in proliferation and apoptosis themselves.

**Table 1** Median Ki67 apoptotic index (AI) in breast carcinomas after 3 months of chemoendocrine therapy (Mitoxantrone + methotrexate + tamoxifen) in relation to the clinical response during that treatment. Some sections that could be analysed for Ki67 had insufficient cells for the analysis of apoptosis

	CR/MRD	PR	NC/PD	P
AI (%)	0.15 (n = 23)	0.28 (n = 20)	0.48 (n = 8)	0.008
Ki67 (%)	1.25 (n = 32)	2.9 (n = 25)	19.6 (n = 12)	0.016

CR, complete response; MRD, minimal residual disease; PR, partial response; NC, no change; PD, progressive disease; n, number of patients.

**Clinical studies**

**Oestrogen deprivation in breast cancer patients**

There are still few data that confirm that endocrine therapy results in apoptosis in human breast cancer. We have demonstrated that modest increases in apoptosis occur with tamoxifen treatment after approximately 14 days, and a more consistent change with the pure anti-oestrogen, ICI 182780 (Ellis *et al.* 1997a). Thus hormonal treatment increases cell death in addition to decreasing cell proliferation in breast tumours in humans. It will be important to determine whether the persistence of the apoptotic changes noted in our xenograft material is also observed in patients.

**Changes in proliferation and clinical response**

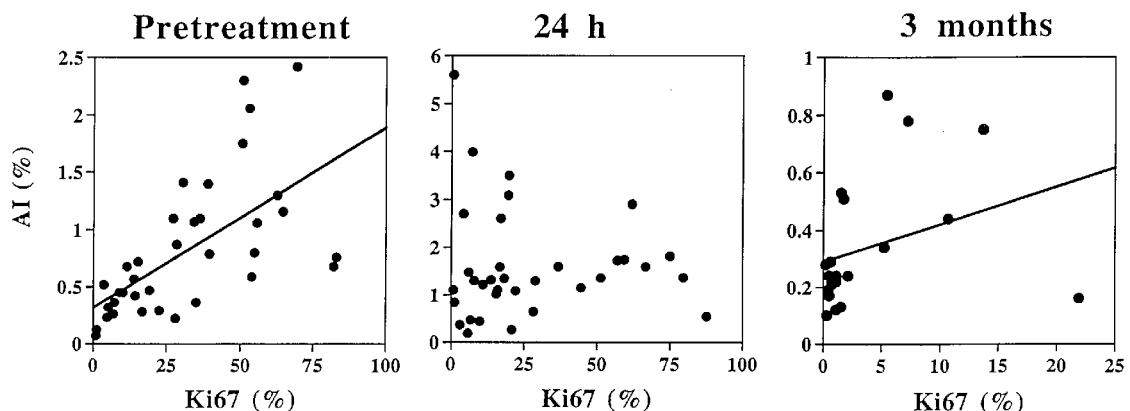
Changes in Ki67 can also be measured in samples taken by fine needle aspiration and prepared as cytopspins

(Makris *et al.* 1997). We have studied changes in Ki67 during tamoxifen therapy, chemotherapy, and chemoendocrine therapy, and have investigated the relationship between the clinical response of patients to changes in proliferation at 14 or 21 days after the start of therapy. In all three cases, there was a highly significant reduction in Ki67 staining amongst the responders, but no significant decrease in the non-responders (Assershon *et al.* 1998).

**Changes in apoptosis during chemotherapy**

We have previously demonstrated that the apoptotic index is significantly increased after 24 h of treatment with chemotherapy (Ellis *et al.* 1997b). We have now confirmed this in a further series of 22 patients. It is possible that this change in apoptosis may predict the clinical response, but it may also be useful as an end-point in itself, either in drug development or in establishing the controlling factors that elicit this change in apoptosis in response to therapy (e.g. in addressing such questions as whether apoptosis is increased by chemotherapy only in those tumours with wild-type p53).

Although apoptosis is increased 24 h after the start of chemotherapy, it has become clear that there is a very substantial decrease in apoptosis by 3 months after the start of chemotherapy (Ellis *et al.* 1998) and that, in the same residual tumours, amounts of Ki67 are also markedly decreased, from a median pretreatment value of 8.0% to a median value of only 1.3%. Figure 2 demonstrates that the close relationship that exists between apoptosis and proliferation before treatment starts is disrupted after 24 h of therapy and that, although there is a statistically significant relationship 3 months into therapy, by that time the large majority of tumours have very low proliferation and apoptotic indices.



**Figure 2** Relationship between proliferation (Ki67) and apoptotic index in human breast carcinomas before and 24 h and 3 months after the start of chemotherapy. The relationships before treatment and at 3 months were statistically significant ( $p=0.723$ ,  $P<0.0001$ ;  $p=0.492$ ,  $P=0.03$  respectively).

The data referred to above, which indicate that those tumours which have the greatest change in proliferation are those which are most responsive to treatment, would suggest that those tumours which continue to show relatively high proliferation and apoptotic indices after 3 months are more likely to be non-responders to treatment. This has been confirmed by the relationships seen after chemoendocrine therapy, in which a significant relationship was seen between the clinical response to treatment and both the level of Ki67 and the apoptotic index, both of which were greater in those patients who showed no response to treatment (Table 1); Wu *et al.* 1996).

## Conclusions

It is clear that increased apoptosis and decreased proliferation are common factors in the biological response of breast cancer to chemotherapy and endocrine therapy. Clinical responses are associated with reduced proliferation during chemotherapy, endocrine therapy and chemoendocrine therapy. These changes may predict response and benefit, can assist in drug development, and also provide appropriate end-points that can be related to other biological predictors of response and resistance.

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