The biology of neoadjuvant chemotherapy for breast cancer

S Cleator¹,², M Parton¹ and M Dowsett¹

¹Academic Department of Biochemistry, Royal Marsden Hospital, Fulham Road, London SW3 6JJ, UK
²Breakthrough Toby Robins Breast Cancer Research Centre, Mary Jean Mitchell Green Building, Chester Beatty Laboratories, Institute of Cancer Research, London SW3 6JB, UK

(Requests for offprints should be addressed to S Cleator; Email: scleator@icr.ac.uk)

Abstract

Neoadjuvant/pre-surgical medical therapy of breast cancer provides a unique opportunity to derive biological information related to tumour response. Large clinical trials of neoadjuvant chemotherapy have established that pathological complete remission is an independent predictor of improved disease-free survival. Clinical response has been found to parallel substantial reductions in the proliferation of breast cancer cells. Increased apoptosis also occurs, but it is not closely associated with response. Numerous biological markers such as p53, bcl-2, oestrogen receptor (ER) and HER2 have been assessed for their possible role in chemoresistance/response, but the data are not clear at this stage. Continuing work using cDNA microarrays may yield new, more reliable indices of likely response and an improved insight into biological processes related to chemotherapeutic response.

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Introduction

Biological rationale for neoadjuvant chemotherapy

Most breast cancer patients who receive chemotherapy do so immediately after surgery – that is, adjuvantly. However, chemotherapy may be given before surgery, as so-called neoadjuvant treatment. Synonymous terms include ‘primary medical therapy’ or ‘induction chemotherapy’.

The use of neoadjuvant chemotherapy originates from the treatment of locally advanced and inoperable breast cancer. In the special case of inflammatory breast cancer, primary chemotherapy occupies a central role, with many centres favouring treatment with cytotoxic agents and radiotherapy, and avoiding surgery if at all possible.

Interest in neoadjuvant chemotherapy in part arose after animal studies conducted by Fisher et al. (1989a, b). In various animal models, they demonstrated that removal of the primary tumour resulted in an increase in the labelling index in residual tumour cells and an increase in circulating growth-stimulating factors (Fisher et al. 1989a). Administration of neoadjuvant chemotherapy, endocrine treatment or radiotherapy to these animals impaired the increase in cell growth observed in residual tumour cells in untreated animals (Fisher et al. 1989b). Furthermore, neoadjuvant treatment prevented the production of circulating factors that were capable of causing stimulation of growth of similar tumours in untreated recipients. On the basis of these results, it was hypothesised that the delivery of chemotherapy before surgical excision of tumour may improve patient outcome by preventing unfavourable kinetic responses.

Clinical rationale for neoadjuvant chemotherapy

Conventionally, neoadjuvant treatments are given to downstage tumours, with the aim of rendering them more amenable to conservative surgery. In the case of breast cancer, this translates to the ability to downstage tumours to a point at which mastectomy can be avoided whilst maintaining an equivalent rate of local control.

This review first considers the outcome of the major clinical studies and then discusses the molecular understanding of the mechanism of action of key cytotoxics of contemporary importance. Thereafter, biomarker studies are considered in relation to the prognostic and predictive information they provide, focusing on the insight into biological mechanisms that they provide.

Clinical experience with neoadjuvant chemotherapy

Randomised trials

Following the demonstration of the feasibility of neoadjuvant chemotherapy in phase II trials, several phase III trials have
been performed to compare mastectomy, local control and survival rates with those of conventional treatment in operable disease. Another objective of these trials was to assess whether the pathological response to chemotherapy could be correlated with outcome; although the opportunity fully to stage these patients pathologically at time of presentation is lost, the extent of residual viable disease after chemotherapy can be evaluated accurately.

The largest of these studies was the multi-centre National Surgical Adjuvant Breast and Bowel Project B-18 (NSABP B-18) trial (Fisher et al. 1997, 1998). Fifteen hundred women were allocated randomly to groups to receive either adjuvant or neoadjuvant chemotherapy with adriamycin and cyclophosphamide (AC). Thirty-six percent of the patients allocated to receive neoadjuvant chemotherapy achieved a complete clinical response (cCR) and 13% had no demonstrable disease on microscopic examination of the surgical sample (complete pathological response, pCR). After a follow up of 5 years, no differences have been observed between the two arms in the incidence of the development of regional or distant relapse. Patients demonstrating cCR were found to have a significantly improved disease-free survival ($P=0.0014$), but not overall survival. In contrast, pCR was associated with improved disease-free survival ($P=0.0001$) and overall survival ($P=0.06$). This has led to pCR becoming a major endpoint in continuing trials, as a potential surrogate for long-term outcome.

The European Organization for Research and Treatment of Cancer recently reported on the results of a trial of very similar design (van Der Hage et al. 2001). Seven hundred patients were allocated randomly to groups to receive either four cycles of neoadjuvant or adjuvant 5-fluorouracil, epirubicin and cyclophosphamide (ECF) chemotherapy. No difference in overall or disease-free survival was seen between the two groups. cCR was seen in 6.6% of the neoadjuvant group, and pCR in only 3.7%. As in the NSABP study, pCR but not cCR was associated with an overall survival benefit, with the $P$ value on the latter in this case reaching statistical significance ($P=0.008$). In the neoadjuvant group, 23% of patients were downstaged to being suitable for breast-conserving surgery from previously requiring mastectomy. These patients did less well in terms of overall survival (hazards ratio 2.53; 95% confidence interval 1.02 to 6.25) compared with patients deemed to be suitable for breast-conserving surgery before chemotherapy although, interestingly, there was no effect on locoregional recurrence rates. The inferior survival of this group may be a reflection of the more aggressive biology of their tumours.


The general consensus from these trials is that neoadjuvant chemotherapy results in a modest reduction in mastectomy rates, possibly accompanied by a small increase in the risk of local recurrence, especially in younger women. Importantly, for the future of presurgical studies, on the basis of the above observations it is possible to say that neoadjuvant chemotherapy is of no detriment to patients in circumstances when adjuvant chemotherapy would be indicated.

### Newer regimens

The AC chemotherapy regimen remains the gold standard in neoadjuvant chemotherapy. However, several trials have reported high response rates for taxane-containing regimens.

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Table 1  Randomised clinical trials of neoadjuvant compared with adjuvant chemotherapy in operable breast cancer

<table>
<thead>
<tr>
<th>Study</th>
<th>Author</th>
<th>Clinical stage (all M0)</th>
<th>Year</th>
<th>No. of patients</th>
<th>Preop. arm treatment</th>
<th>Postop. arm treatment</th>
<th>DFS preop. (%)</th>
<th>DFS postop. (%)</th>
<th>$P$</th>
<th>OS preop. (%)</th>
<th>OS postop. (%)</th>
<th>$P$</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSABP-B18</td>
<td>Fisher et al.</td>
<td>T1–3</td>
<td>1997</td>
<td>1523</td>
<td>AC</td>
<td>AC</td>
<td>67</td>
<td>67</td>
<td>NS</td>
<td>80</td>
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<td>NS</td>
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<tr>
<td></td>
<td>Fisher et al.</td>
<td>N0–1</td>
<td>1998</td>
<td></td>
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<tr>
<td></td>
<td>Van Der Hage et al.</td>
<td>T1c–T4b</td>
<td>2001</td>
<td>698</td>
<td>FEC</td>
<td>FEC</td>
<td>65</td>
<td>70</td>
<td>NS</td>
<td>82</td>
<td>84</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>RMH</td>
<td>Powles et al.</td>
<td>T0–T4</td>
<td>1995</td>
<td>309</td>
<td>2M/3M + tam.</td>
<td>2M/3M + tam.</td>
<td>N/A</td>
<td>N/A</td>
<td>NS</td>
<td>N/A</td>
<td>N/A</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Makras et al.</td>
<td>N0–1</td>
<td>1998</td>
<td></td>
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<tr>
<td>Institute</td>
<td>Mauriac et al.</td>
<td>T2–T3</td>
<td>1991</td>
<td>272</td>
<td>EVM × 3, MTV × 3</td>
<td>EVM × 3, MTV × 3 if high risk</td>
<td>50</td>
<td>50</td>
<td>NS</td>
<td>82</td>
<td>75</td>
<td>NS</td>
<td>Preop. arm: underwent RT or Sx</td>
</tr>
<tr>
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<td>Mauriac et al.</td>
<td>N0–1</td>
<td>1999</td>
<td></td>
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<tr>
<td>Institute</td>
<td>Scholl et al.</td>
<td>T2–T3</td>
<td>1994</td>
<td>414</td>
<td>CAF</td>
<td>(RT ± Sx)</td>
<td>N/A</td>
<td>N/A</td>
<td>NS</td>
<td>65</td>
<td>60</td>
<td>NS</td>
<td>Sx if incomplete response to chemo/RT</td>
</tr>
<tr>
<td>Institute</td>
<td>Broet et al.</td>
<td>N0–N1</td>
<td>1999</td>
<td></td>
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<tr>
<td>Institute</td>
<td>Petersburg</td>
<td>T1–2N2</td>
<td>1994</td>
<td>271</td>
<td>1–2 × TFM RT → Sx</td>
<td>RT → Sx → TFM + Sx→ M0 × 4–5</td>
<td>81</td>
<td>72</td>
<td>0.04</td>
<td>86</td>
<td>78</td>
<td>NS</td>
<td></td>
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</tbody>
</table>

**Notes:** DFS, disease free survival; OS, overall survival; AC, adriamycin, cyclophosphamide; CAF, adriamycin, fluorouracil, cyclophosphamide; EVM, epirubicin, vincristine, methotrexate; FEC, 5-fluorouracil, epirubicin, cyclophosphamide; 2M/3M, mitoxantrone, methotrexate/mitoxantrone, methotrexate, mitomycin C; MTX, methotrexate, thiotepa, vindesine; TFM, thiotepa, fluorouracil, methotrexate; N/A, not available; RT, radiotherapy; Sx, surgery; tam., tamoxifen; NS, $p>0.05$. 

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*S Cleator et al.: Biology of breast neoadjuvant chemotherapy*
(Holmes et al. 1991, Moliterni et al. 1997), although phase III studies have failed to demonstrate an advantage over anthracyclines (Buzdar et al. 1999).

It is known that taxanes have activity in anthracycline-resistant disease, which prompted the design of the NSAPB B-27 study. This is a three-arm trial in which 2411 patients have been allocated randomly to (1) four cycles of neoadjuvant AC followed by surgery, (2) four cycles of AC followed by four cycles of docetaxel followed by surgery, and (3) four cycles of AC, followed by surgery and then adjuvant docetaxel. Preliminary results from the trial demonstrate that AC + docetaxel is associated with a statistically significant greater rate of pCR than AC alone (18.7% compared with 9.8%) and a statistically significant greater rate of pathologically negative nodes at time of surgery (58.1% compared with 50.7%) (NSABP 2001). As yet, the impact of this on survival is unknown.

In another study that included some individuals with locally advanced disease, patients received neoadjuvant cyclophosphamide, doxorubicin, vincristine and prednisolone (CVAP). Non-responders after four cycles proceeded to four cycles of docetaxel and responders were allocated randomly to either four further cycles of CVAP chemotherapy or four cycles of docetaxel. An increased pCR rate was observed in those responders who switched to docetaxel, although docetaxel did not result in a reduction in the rate of axillary node involvement (Smith et al. 2002).

Neoadjuvant chemotherapy: mechanism of cytotoxic action

Background

Patients undergoing breast neoadjuvant chemotherapy provide an ideal research tool for the investigation of factors that may be used as indicators of outcome (prognostic factors) or indicators of chemoresponse (predictive factors). The breast is anatomically accessible and methods exist for obtaining serial biopsies atraumatically and in a manner that is acceptable to the patient. This provides an opportunity to observe the effects of chemotherapy on biological processes. As response to chemotherapy can also be prospectively assessed readily in this setting (an opportunity that never presents itself in the adjuvant setting), the relationship between the changes in the biological processes and the response can be determined. Because changes in proliferation or apoptosis are required for change in tumour growth, studies of these processes and their control pathways are of particular interest (Fig. 1, Fig. 2).

Mechanism of action of cytotoxic agents used in neoadjuvant chemotherapy treatment

The two classes of cytotoxic drugs that demonstrate most activity towards breast cancers are anthracyclines and taxanes. Experience over the years has shown that combination chemotherapy is more effective than single agents. The combination approach may succeed where single agents fail probably because, although individually the maximum tolerated doses of each drug may be insufficient to kill all malignant cells, in combination 100% kill may be achieved with tolerable toxicity, as long as the toxicity of the component drugs is non-overlapping. Furthermore, tumours represent a heterogeneous population of cell lineages that vary in terms of sensitivity (and resistance) to chemotherapy. A combination approach allows a broader range of coverage. Of the many chemotherapy regimens that have been used in the treatment of breast cancer, the most widely prescribed will be discussed briefly.

Anthracyclines

The most commonly used anthracycline, doxorubicin, is a natural compound produced by the Streptomyces species, whereas epirubicin, which is also widely used in the treatment of breast cancer, is synthetically produced. They act by several mechanisms, but most important is their interaction with the nuclear enzyme topoisomerase II (Smith & Soues 1994). In the resting state, DNA is tightly coiled into a compact secondary structure. Topoisomerases reduce DNA twisting and supercoiling, allowing selected regions of DNA to untangle and so engage in transcription, replication or repair processes.

Anthracyclines induce formation of covalent topoisomerase I/O–DNA complexes, which prevent the enzyme from completing the religation part of the ligation–religation reaction (Chen & Liu 1994). Anthracyclines are also DNA intercalators, causing single-stranded or double-stranded breaks. Furthermore, they can undergo reduction to produce free radicals that can cause oxidative damage to cellular proteins (Smith & Soues 1994).
Taxanes
The first taxane, paclitaxel, was isolated from the bark of the Pacific Yew, *Taxus brevifolia*, in 1971. Since then, a similar compound, docetaxel, was isolated from more abundant species of yew and is now generated semi-synthetically.

Taxanes have been shown to bind to polymerised microtubules (Parness & Horwitz 1981), resulting in a shift in the equilibrium between tubulin dimers and microtubules, towards the latter. As a result microtubules are stabilised against depolymerisation, causing microtubule bundling in cells (Schiff & Horwitz 1980, Rowinsky et al. 1988). This prevents the microtubules from performing the functions necessary to facilitate mitosis, resulting in a sustained mitotic block (Jordan et al. 1993).

It has also been demonstrated that taxanes cause immune modulation via an increase in cytokines such as TNFα (Burkhart et al. 1994) and inhibition of angiogenesis (Sollott et al. 1995, Belotti et al. 1996).

Combination chemotherapy
An overview of breast cancer chemotherapy confirmed the superiority of combination chemotherapy over single agents (EBCTCG 1992). The chemotherapy regimen most rigorously assessed consists of cyclophosphamide, methotrexate and 5-fluorouracil. Cyclophosphamide, an alkylating agent, alkylates DNA, which leads to formation of crosslinks. Methotrexate is a folate antagonist. It acts by inhibiting the reduction of folate, which is required for the synthesis of a variety of essential coenzymes. In particular, it causes inhibition of thymidylate synthetase, so inhibiting DNA and RNA synthesis. 5-Fluorouracil, a fluoropyrimidine, acts by inactivating thymidylate synthetase, so blocking DNA synthesis (Dutch et al. 2000).

Response and resistance to chemotherapy
General
Defective apoptosis and, to a degree, inappropriate proliferative activity, underpin the process of tumorigenesis (Johnstone et al. 2002). It is important to emphasise that, despite variations in individual mechanism of action, all the above agents bring about cancer cell death by activation of the apoptotic cascade as the principal mechanism of chemotherapy-induced cancer cell death (Hickman et al. 1992). As will be discussed, evidence indicates that defective apoptosis is probably the basis of chemotherapeutic resistance in many
cases. Study of the changes in the components of the apoptotic and proliferative pathways in breast cancers during chemotherapy may yield information about the molecular pathways associated with chemotherapy response and resistance.

There are very few studies that have set out to study the biology of neoadjuvant chemotherapy in breast cancer, and those that exist are limited in the number of time points. However, numerous studies have tried to identify markers or changes in markers that are associated with response or resistance to treatment, with the goal of introducing them as predictive markers. Areas of key importance are:

- What changes occur to underpin the regression of tumours?
- What biological processes are required to facilitate the changes and which, if deficient, may lead to resistance?
- Are there identifiable features in the cells that remain at the end of chemotherapy, which allow their survival?

Predictive markers in the setting of neoadjuvant chemotherapy have been assessed in terms of correlation with clinical or pathological response, on the basis that clinical and pathological responses to treatment are surrogates of disease-free and overall survival respectively (Fisher et al. 1998, van Der Hage et al. 2001). It should be noted therefore that, for full validation, biological markers require that a direct correlation with patient outcome be demonstrated.

Changes in proliferation and apoptosis caused by chemotherapy

Chemotherapy, radiotherapy and, in part, hormonal treatments act by inducing apoptosis (Hickman 1992, Ellis et al. 1997b, Verheij & Bartelink 2000). Apoptosis, or programmed cell death, is an active process controlled by several regulators that initiate or inhibit activity (Hengartner 2000). It is characterised morphologically at the cellular level by membrane blebbing, cell shrinkage, protein fragmentation, chromatin condensation and DNA degradation. There then follows a rapid engulfment of the remainder by neighbouring phagocytes. In normal development and growth, apoptosis is a tightly regulated mechanism that can potentially lead to multiple pathology, including carcinogenesis (Reed 1999), if dysregulation occurs. The balance between proliferation and apoptosis is crucial in determining the overall growth or regression of the tumour (Reed 1999, Tammi et al. 2001). Given that changes in apoptosis and proliferation are ultimately involved in the response process, these parameters of cell growth are suitable candidates for the study of predictors of response. The pathways leading to apoptosis and proliferation are inextricably linked at various points, for example by the tumour suppressor gene, p53 (Eastman & Rigas 1999).

Detection and quantification of apoptosis in breast cancer

The ‘gold standard’ for detection and quantification of apoptotic cell death in situ has been morphological assessment with either electron microscopy or light microscopy (Kerr et al. 1994). However, this is often difficult and time consuming, even for trained histopathologists, and therefore a variety of methods have been developed for the identification of these cells. The most widely used techniques are in situ end labelling (Wijsman et al. 1993) and terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay (Gavrieli et al. 1992) (Fig. 2). Both these methods involve incorporation of labelled nucleotides at the free 3′ OH end of DNA, including the 3′ ends produced by endonuclease action during apoptosis. Cell morphology can be examined simultaneously with these techniques, and infrequent events are more readily identified. Other methods of detecting apoptosis include characterisation of the membrane changes that occur during apoptosis (annexin V) or detection of release of enzymes (tissue transglutaminases). It must be recognised that most of these methods provide only a snapshot of a dynamic process, and that apoptotic bodies are removed rapidly by surrounding macrophages as they arise.

Detection and quantification of proliferation

A variety of methods have been used to evaluate proliferation in breast carcinomas, some of which depend on the cell cycle phase that they measure. Mitotic index, a component of the histopathological tumour grading system, measures the proportion of the cells in the mitosis phase (M) of the cell cycle only. Detection of cells synthesising DNA (i.e. in S-phase) can be evaluated using the thymidine labelling index (Silvestrini et al. 1985) and labelling with bromodeoxyuridine, a halogenated analogue of thymidine (Thor et al. 1999). However, these materials require fresh material of sufficient quantity. Another method detects the percentage of cells in S-phase. The S-phase fraction, measured by DNA flow cytometry, uses fresh, frozen or paraffin-embedded tissue. Immunohistochemical methods utilising the Ki67 monoclonal antibody to determine cell proliferation are widely accepted. The Ki67 antibody reacts with a nuclear antigen that is expressed in G1, S, G2 and mitosis, but not in G0 (resting phase) (Gerdes et al. 1984), but its use is restricted to frozen tissue (Fig. 2). MIB-1 is a murine monoclonal antibody against recombinant parts of the Ki67 antigen, and can be used in formalin-fixed, paraffin-embedded tissue sections. A good correlation between Ki67 antibody and MIB-1
measurement has been reported (Querzoli et al. 1996) ($r = 0.73, P < 0.001$).

**Apoptosis and proliferation as prognostic/predictive markers**

Apoptosis and proliferation markers have been studied in breast cancer extensively, as both prognostic and predictive markers. In untreated breast cancer, apoptosis correlates strongly with proliferation (Lipponen et al. 1994, Berardo et al. 1998, Ellis et al. 1998, van Slooten et al. 1998, Zhang et al. 1998). Thus the cell population of highly proliferative tumours appears to also be lost at a high rate. However, the evidence for apoptosis as an independent prognostic marker appears to be largely inconclusive (Lipponen et al. 1994, Berardo et al. 1998, Zhang et al. 1998). High apoptosis is associated with tumours of high grade, HER2 expression and p53 overexpression, and is inversely associated with Bcl-2 and ER (Lipponen et al. 1994, Hori et al. 1997, Rochaix et al. 1999, Liu et al. 2001). Therefore a poor outcome for such tumours is expected. Despite comprehensive large studies (>700) examining the relationship between apoptosis and disease-free and overall survival, apoptosis failed to emerge as an independent prognostic marker in patients with node-negative or node-positive breast cancer (Liu et al. 2001). In contrast, tumours of high proliferation as detected by Ki67 or MIB-1 have largely been associated with decreased disease-free and overall survival (Pinder et al. 1995, Brown et al. 1996, Raïlo et al. 1997, Wintzer et al. 1991). Interestingly, this is despite the widely reported positive relationship between level of cell proliferation and response to chemotherapy in the neoadjuvant setting, supporting pre-clinical observations of high proliferation and chemosensitivity (Bonetti et al. 1996, Chevillard et al. 1996, MacGrogan et al. 1996).

Biological studies during neoadjuvant treatment have revealed that a measurable increase in apoptosis occurs in breast tumours within 24 h after the start of chemotherapy (Ellis et al. 1997c, Archer et al. 1999). This was also associated with a concomitant decrease in proliferation (Archer et al. 1999). A small pilot study by Chang et al. (1999) using serial fine needle aspirations (FNAs) and flow cytometry demonstrated a significant relationship between early changes in apoptosis (24–48 h) and eventual clinical response (Chang et al. 1999). A later, more extensive study using more rugged methodology failed to confirm the relationship between early changes in apoptosis and proliferation with response (C D Archer, personal communication) (Parton et al. 2001). Decreases in proliferation are relatively modest after 24 h, but by 21 days are more extensive, and have also been shown to correlate with a favourable response (Makris et al. 1998a, Chang et al. 1999). Thus it appears that timing of subsequent biopsy or FNA is pivotal in demonstrating a relationship between significant changes as a result of treatment-associated cell death and clinical response; this in turn may vary between tumours and mode of action of chemotherapy. Nonetheless, the relationship between reduced Ki67 or increased apoptosis is not close at any time point examined. In addition to variable kinetics between tumours, this may result from the need for a greater change in these parameters to lead to regression in a fast-growing tumour than is required in a slowly progressing tumour. This becomes clear from examination of Fig. 3, in which tumour regression/progression are seen in MCF-7 xenografts during oestrogen withdrawal or tamoxifen treatment. Parallel measurements of Ki67 and the apoptotic index showed that twofold reductions or threefold increases, respectively, were associated with only stable disease in this fast-growing tumour. Lesser changes would be associated with continued progression but, in a slowly growing tumour, could result in regression. Although these data pertain to endocrine treatment, similar principles should hold for chemotherapy.

In residual tissue at the cessation of chemotherapy, both apoptosis and proliferation are significantly reduced (Ellis et al. 1998); thus, in resistant cell populations at the end of treatment, the balance between apoptosis and proliferation is maintained. This raises the question of whether the decrease in proliferation is the result of downregulation in the entire cell population by a triggered ‘switching off’ of proliferative regulators by treatment, or reflects a selection of residual, less proliferative cells that are intrinsically less sensitive to chemotherapy and have been preserved throughout treatment. The latter concept is in keeping with the finding that the residual cell population has greater concentrations of detectable Bcl-2 protein, an inhibitory apoptosis regulator (Ellis et al. 1998). However, it is simplistic to consider that Bcl-2 may be the sole factor related to this reduced proliferation, as Bcl-2 is a member of an extensive family of regulators of apoptosis. Honkoop et al. (1998) were able to demonstrate that a high proliferative index in residual tumour was associated with decreased survival. Interestingly, this may be reflected in the finding by Vakkala et al. (1999) that increased proliferation and reduced apoptosis in the recurrent breast tumours in comparison with the primary lesion also predicted for a worse prognosis.

**The biological basis of drug resistance in breast cancers**

**General**

In the large neoadjuvant studies discussed above, the majority of patients harboured residual viable tumour cells at time of surgery after completing chemotherapy (Fisher et al. 1997, van Der Hage et al. 2001). Invasive or acquired chemoresistance is therefore a common occurrence in the setting of neoadjuvant breast cancer treatment. Disabling of apoptosis is a central event in tumour development, and most
chemotherapeutic drugs require functioning apoptotic pathways to induce cell death (Johnstone et al. 2002). This provides a possible explanation of why drug-naive tumours can be resistant. Drug resistance can also be acquired, as is demonstrated through the common clinical observation that chemotherapy resistance is even more evident in the setting of second- or third-line treatment. Cytotoxic drugs can be mutagenic and, in the context of cells that have defective apoptotic pathways, it is possible that such induced mutations might occur, resulting in resistance to other drugs. The fact that chemically unrelated drugs can kill tumours by activating a common pathway means that single mutations may lead to resistance to a range of drugs (see below).

‘Classical drug resistance’

A significant component of anthracycline resistance is enhanced efflux of drug via the P-glycoprotein pump, which is encoded by multidrug-resistant (MDR) genes (Kartner et al. 1985). At present, the best-described mechanism of resistance to tubulin-binding agents is also the MDR phenotype (Ling 1992). Evidence is now emerging that the P-glycoprotein pump can cause drug resistance much further down the apoptotic pathway, by inhibition of caspases (Johnstone et al. 2000), downstream effectors of apoptosis.

It has been recognised for some years that glutathione upregulation represents another common mechanism of resistance. This antioxidant is believed to give rise to resistance by providing protection against free-radical damage (Lutzy et al. 1989). Investigation of these possible pathways of resistance in neoadjuvant studies has not been reported.

Molecular defects

A panel of biological markers including regulators such as p53, Bcl-2 family proteins, caspases and DNA fragmentation factor have been described as having a role in apoptosis. The assessment of these in cell lines and in clinical samples, particularly in the neoadjuvant setting, has helped build a picture of their contribution to the biology of chemoresistance.

Apoptotic pathways

p53

DNA damage and other cellular insults are detected by Chk2 and the product of the ataxia telangiectasia mutated gene (ATM), resulting in activation of p53 (Khanna & Jackson 2001). This triggers transcriptional activation of Bax and other pro-apoptotic Bcl-2 family members and repression of Bcl-2 and other anti-apoptotic genes. Loss of p53 disrupts apoptosis and accelerates tumour development in transgenic mice (Attardi & Jacks 1999). There is evidence from in vitro (Lowe et al. 1993) and animal studies (Lowe et al. 1994) that defective p53 is associated with resistance to chemotherapy. Furthermore, loss of p53 function correlates with multidrug resistance in many tumour types (Wallace-Brodeur & Lowe 1999).

p53 mutations are common in breast malignancies (Andersen et al. 1993) and are associated with a poor prognosis (Overgaard et al. 2000). Mutant p53 is characterised by a long half-life, leading to nuclear accumulation. Thus immunohistochemical staining of p53 is commonly taken to indicate the presence of mutant p53. However, not all p53 mutations cause increased staining (Aas et al. 1996). Several

Figure 3 Clinical response and relationship with apoptotic and proliferative features of tumours. Data are plotted as straight lines for illustrative purposes. Original data relate to MCF-7 xenografts in nude mice (Johnston et al. 1999). AI, apoptotic index; E2, oestradiol.
neoadjuvant studies have failed to detect a predictive value to p53 staining with regards to chemoresponsiveness in breast cancers (MacGrogan et al. 1996, Niskanen et al. 1997, Bonetti et al. 1998, Rozan et al. 1998). Work by Geisler et al. (2001) has demonstrated that specific p53 mutations are associated with resistance to doxorubicin in the neoadjuvant setting. A number of these mutations were not associated with enhanced staining for p53, which would explain why immunohistochemical studies have been inconclusive. Assessment of p21 (WAF1/cip1) staining as a downstream marker of p53 activity improved the relationship between p53 staining and mutational status, but was not an absolute predictor of functional p53 (Ellis et al. 1997c). The same group of investigators also demonstrated a strong correlation between p53 mutation and HER2 expression.

In another study, p53 staining and mutations were studied in relation to the response of 67 breast tumours to neoadjuvant 5-fluouracil epirubicin cyclophosphamide (FEC) or docetaxel chemotherapy. In the FEC group, treatment failure was associated with p53 mutation and absence of apoptotic cells after treatment. For paclitaxel treatment, in which the role of p53-mediated cell death is less clear (Lanni et al. 2001), a response was supported by deficient p53 and normal p53 was associated with resistance (Kandiolser-Eckersberger et al. 2000).

**Bcl-2 family**

Bcl-2 is one of a family of cytoplasmic proteins that are regulated by p53 and phosphorylation by tyrosine kinases. Bcl-2 and Bcl-XL inhibit apoptosis, whereas Bax, Bag-1 and Bad promote it. Bcl-2 is overexpressed in approximately 80% of breast tumours (Krajewski et al. 1999) and correlates with low proliferative indices, high oestrogen receptor (ER) content, absence of p53 and HER2 staining, and good outcome (van Slooten et al. 1996). Bcl-2 overexpression is classically associated with follicular lymphomas and appears to be responsible for the chemoresistance exhibited by these tumours (Reed 1997). The situation in breast cancer is less clear-cut. In the randomised study conducted by van Slooten et al. (1996) referred to above, Bcl-2 had no predictive value for a single cycle of preoperative FEC chemotherapy (van Slooten et al. 1996). Other non-randomised neoadjuvant studies have also failed to show any predictive value of Bcl-2 in terms of chemoresponse (Frassoldati et al. 1997, Collecchi et al. 1998, Ellis et al. 1998). However, Bonetti et al. (1998) found that, in a series of women with advanced breast cancer, Bcl-2 negativity was associated with increased response rate to neoadjuvant chemotherapy; however, their numbers of patients were very small. As mentioned previously, the finding that residual tumour at cessation of chemotherapy has increased Bcl-2 concentrations relative to pre-chemotherapy specimens accords with the concept of a role of Bcl-2 expression in tumour resistance (Collecchi et al. 1998, Ellis et al. 1998).

Overexpression of Bcl-XL, a related member of the Bcl-2 family, promotes chemotherapy resistance of mammary tumours in a syngeneic mouse model (Liu et al. 1999), but clinical data are currently lacking. In contrast, reduced staining for Bax, a proapoptotic regulator, is associated with poor breast cancer prognosis in the setting of metastatic disease (Krajewski et al. 1995). Furthermore, in this and a further metastatic study (Sjostrom et al. 1998), response to combination chemotherapy was significantly worse in tumours with reduced Bax.

**Other defects in apoptosis involved in drug resistance**

Functional mutations in many p53 upstream regulators and downstream effectors occur in human tumours. Apaf-1 is downstream to the Bcl-2 family, and required for activation of caspase-9 activation. Loss of Apaf-1 expression in human leukaemia and ovarian cell lines is associated with a decrease in drug-induced apoptosis, which can be re-established after transfection with Apaf-1 (Jia et al. 2001).

Altered function of upstream regulators (ATM and Chk2) and downstream effectors and regulators (PTEN, heat-shock proteins, inhibitor of apoptosis proteins (IAPs), caspase-8) occurs in tumours and, in many cases, correlates with drug resistance (Johnstone et al. 2002).

Although this information is likely to be important to our understanding of the probable underlying mechanisms of drug resistance in breast cancer, assessment of these defects in neoadjuvant studies of the type described above is still awaited, to assess their clinical significance.

**Proliferative pathways**

**Steroid receptor status**

There are data that suggest that steroid receptor negativity predicts for chemosensitivity. In one recent study, tumours negative for ER or progesterone receptor demonstrated clinical response and pCR rates superior to that of neoadjuvant chemotherapy (various regimens) superior to that of ER positive tumours (Colleoni et al. 2000). This is also supported by adjuvant/perioperative data from the same centre (Colleoni et al. 2001). Several other studies have demonstrated a statistically significant greater response rate to neoadjuvant chemotherapy in steroid receptor-negative patients (Bonadonna et al. 1990, MacGrogan et al. 1996, Daidone et al. 1999).

The underlying mechanism by which lack of ER sensitises cells to apoptosis by chemotherapy is not fully established, but in vitro studies suggest that ER signalling can increase levels of Bcl-2 and induce anthracycline resistance (Teixeira et al. 1995).

**HER2 (c-erbB-2, c-neu)**

HER2 is a transmembrane receptor similar to epidermal growth factor receptor that is overexpressed in about 25% of
breast tumours (Slamon et al. 1989) and associated with poor outcome (Menard et al. 2001). Activation of HER2 induces activation of ras, leading to a phosphorylation cascade ultimately resulting in cell proliferation.

Despite some earlier positive studies, recently reported results suggest that stable transfection of normal human mammary epithelial cells with multiple copies of HER2 does not result in changes in sensitivity to a wide variety of chemotherapy agents (Orr et al. 2000).

Clinical data suggest that HER2 overexpression may affect drug sensitivity. Most data have been derived from adjuvant clinical trials in which the outcome of HER2-positive and HER2-negative patients (as assessed by immunohistochimistry) receiving adjuvant chemotherapy has been compared with HER2-positive and HER2-negative patients receiving no treatment or treatment with drugs at lower doses. The majority of studies suggest that HER2-negative, but not HER2-positive, patients derive benefit from chemotherapy based on alkylating agents (Allred et al. 1992, Gusterson et al. 1992, Miles et al. 1999), and other adjuvant studies have shown that overexpression of HER2 is associated with relative sensitivity to anthracycline regimens (Paik et al. 1998, Thor et al. 1998). These data have not been corroborated by results from studies assessing the predictive value of HER2 status in the metastatic setting (Revillion et al. 1996, Niskanen et al. 1997).

The optimal setting for assessing the response rates to chemotherapeutic agents is the neoadjuvant, for reasons given above. A recent study by Petit et al. (2001) demonstrated that HER2 overexpressers responded better to FEC100 than to FEC50, where the number denotes the dose of epirubicin (in mg m−2), whereas HER2-negative patients responded equally to both regimens. Furthermore, HER2-positive patients responded better to FEC100 than did HER2-negative patients (P=0.07). A study published by Geisler et al. (2001) found that HER2 overexpression predicted resistance to a low-dose, weekly doxorubicin schedule, and a study by Willsher et al. (1998) similarly found a correlation between HER2 expression and resistance to neoadjuvant mitoxantrone, methotrexate and mitomycin C. In contrast, some studies of this kind have failed to show a predictive value of this marker (MacGrogan et al. 1996, Rozan et al. 1998, Vargas-Roig et al. 1999, Vincent-Salomon et al. 2000). Thus, at present, the data in neoadjuvant studies are conflicting with respect to HER2 status and response to anthracyclines.

To date, the studies correlating HER2 status and response to taxanes have been small and inconclusive, as reviewed recently (Yamauchi et al. 2001).

**Directions for research**

Resistance to chemotherapy remains a major challenge in the treatment of breast cancers. Some tumours do respond to chemotherapy and patients benefit from it. However, as yet there is no reliable means of predicting chemotherapy responsiveness. Even in those patients who respond, pCR is rare, indicating a sub-population of chemoresistant cells. Most drugs in common usage require an intact apoptotic mechanism. Cancers, however, often arise or at least persist as a consequence of abnormal functioning of this pathway.

Serial biopsies obtained from breast cancers during neo-adjuvant chemotherapy provide a valuable opportunity to observe the pathways involved in cell death over time. Cells remaining in poorly responsive tumours at time of surgery are de facto resistant to treatment and are the cells responsible for treatment failure. Their molecular characterisation may provide an improved understanding of the mechanisms of chemoresistance and lead to new, targeted treatment strategies, possibly in conjunction with current treatments. The data described above provide only a first glimpse of the biology of chemotherapy in primary tumours, but reveal the potential for further studies of this type to provide additional valuable information. Comparisons of other components of the apoptotic and proliferative pathways in responding and non-responding cells may provide further mechanistic information and provide the basis for more rational treatments and better drug development.

The new technologies of comparative genomic hybridisation, real-time polymerase chain reaction, expression microarray and proteomics will be useful in performing these comparisons. The feasibility of these approaches has been demonstrated (Assersohn et al. 2002, Sotiriou et al. 2002), and data from such studies are eagerly awaited.

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