The paradox of cancer cell apoptosis

Bruce C Baguley

Auckland Cancer Society Research Centre, Faculty of Medical and Health Sciences, The University of Auckland, Private Bag 92019, Auckland 1142, New Zealand

TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Measurement of the rates of in vivo apoptosis in tumours
4. Tumour cell turnover
5. The existence of more rapidly growing cell populations in human tumours
6. The existence of a slowly growing tumour cell population in human tumours
7. The nature of dormant tumours
8. Nature of the transitions between tumour populations
9. Examination of the effects of antitumour therapy
10. Perspective
11. Acknowledgement
12. References

1. ABSTRACT

Resistance to apoptosis is an accepted property of human cancer cells and resistance to cancer therapy is often considered to involve increased resistance to apoptosis. However, comparison of the potential doubling times of human tumour cells with the volume doubling times of the tumour from which they are derived implies a high rate of apoptosis. For at least some cancer types, increased proliferation rate and correspondingly increased apoptosis is associated with a poor prognosis. How can resistance to apoptosis and apoptosis be reconciled? One possible resolution of this paradox is that at least two tumour cell populations are involved, a smaller, more rapidly growing population with self-renewal properties and resistance to apoptosis, and a larger, more slowly growing population that is susceptible to apoptosis. The progeny of smaller population thus maintains the larger population. This review describes the evidence for such a model and its implications for cancer therapy.

2. INTRODUCTION

Apoptosis, the process by which cells in the body can be eliminated without causing an inflammatory response, is essential not only for embryogenesis but for normal tissue homeostasis. It allows unneeded cells to be converted by cellular blebbing and fragmentation into an array of smaller bodies, each of which has both an intact plasma membrane and an energy supply to maintain the integrity of its membrane. Apoptotic bodies express signals on their surface to promote their efficient recognition and removal by phagocytic cells in the tissue. They may also have an anti-inflammatory and immunosuppressive effect, contributing to the efficiency of processes such as wound healing (1,2). As shown in Figure 1, there are two major pathways to the induction of apoptosis. In the extrinsic pathway, stimulation of external surface receptors by signalling ligands such as FASL and TRAIL leads to activation of a pathway to apoptosis through a cascade of caspase signalling. In the intrinsic pathway, changes in mitochondrial membrane-associated...
The paradox of cancer cell apoptosis

Figure 1. Simplified diagram of pathways to apoptosis and mechanisms of resistance to apoptosis in tumour cell lines. Caspase-3 mediated apoptosis is thought to be one of the main pathways and is activated by caspase-8 in the extrinsic pathway and by caspase-9 in the intrinsic pathway. The caspase-9 pathway is activated by cytochrome C and other products resulting from a permeability transition of the outer mitochondrial membrane in conjunction with the apoptosome component Apaf-1. The extrinsic pathway is also linked to the intrinsic pathway through the bcl2 family member bid, which promotes the mitochondrial permeability transition. Tumour cells may have increased resistance to apoptosis by the extrinsic pathway through overexpression of FLIP or reduced expression of CD95, DR4 or DR5 receptors. They may also increase resistance by overexpression of inhibitors of apoptosis (IAPs), which act on caspase-9 and caspase-3, or by altered expression of the bcl2 family members (3).

proteins including those of the bcl2 family result in a permeability transition of the mitochondrial outer membrane, leading to release of proteins such as cytochrome C from their storage position between the inner and outer mitochondrial membranes and activation of the caspase signalling cascade (3). Resistance to apoptosis is regarded as one of the five hallmarks of cancer (4) and tumour cells have been demonstrated to exhibit resistance to apoptosis in different ways, as illustrated in Figure 1.

3. MEASUREMENT OF THE RATES OF IN VIVO APOPTOSIS IN TUMOURS

If cancer cells are indeed resistant to apoptosis, do human cancers generate significant numbers of apoptotic cells in vivo? One approach to answering this question is to take tumour biopsy sections and quantitate the frequency of apoptotic cells. A common method of identify apoptotic cells is from the number of broken DNA ends, which increase substantially when a caspase-activated DNAse is present in the nucleus (5). The DNA end-labelling assay (TUNEL assay) provides an “apoptotic index” that has a frequency in humans of about 1% (6). However, the apoptotic index does not provide information on the rate of apoptosis; to do this, one can measure both the average rate of proliferation of individual cells and the rate of tumour growth. A difference between these two rates will indicate the rate of cell loss or turnover. Net tumour growth can be measured by direct volume measurement or computerised tomography (CT) imaging, which typically provides estimates of volume doubling times of several months (7). The proliferation rates of individual tumour cells cannot be determined from single measurement and methods to measure them must involve comparison of some cell cycle characteristics of a tumour population at two different times.

The potential doubling time (Tpot) is the doubling time of a proliferating cell population if one ignores loss of cells from this population. The most commonly used method of estimating Tpot of tumour cells in cancer patients is based on labelling of replicating DNA (i.e. the S-phase population) with a thymidine analogue such as bromodeoxyuridine or iododeoxyuridine (8). These analogues have short plasma half-lives and therefore label cells within a few minutes of administration. It is necessary to take a surgical biopsy of the tumour several hours later to provide the second time point. Disaggregation of the biopsy and analysis by flow cytometry uses a combination of a fluorescent stain such as propidium iodide to stain total DNA and a fluorescent antibody to identify the bromodeoxyuridine (or
iododeoxyuridine) labelled DNA. The antibody identifies the S-phase cells existing at the first time point while the DNA stain identifies the S-phase cells (from their intermediate cellular DNA content) at the second time point. The cell cycle distribution of the antibody-labelled population provides a dynamic picture of the cell cycle time; it shows the rate at which the original S-phase cell population has moved to other phases of the cell cycle during the time between administration of the thymidine analogue and the removal of the biopsy; from this it is possible to estimate the S-phase transit time. The propidium staining provides the proportion of cells in S-phase at the time of the biopsy, and in combination with the S-phase transit time (basically the transit time divided by the S-phase proportion and multiplied by a factor that takes exponential growth into account) provides the estimate of Tpot (9). This technique has been used for many different human tumours, providing Tpot values that vary over a range from 2 days to several weeks with a median of approximately one week. Several studies have shown that Tpot values are related to clinical outcome; at least for some tumour types high proliferation rates, corresponding to short Tpot values, are related to poor prognosis (10,11).

Measurement of Tpot by administration of thymidine analogues is now used infrequently in human studies because these analogues are potential mutagens and the technique requires a surgical procedure (tumour biopsy) that has a small but finite health risk. An alternative method of estimating Tpot, developed in this laboratory (12,13) is to culture tumour material obtained during standard surgery. Partially disaggregated cells from such tissue are cultured for a fixed time (7 days) in the presence or absence of an inhibitor of cell division (typically paclitaxel) and the relative proportions of S-phase cells in the treated and untreated tumour cultures are compared near the end of the incubation period by incorporation of 3H-labelled thymidine into DNA. The number of S-phase cells in control cultures increases with time because of proliferation, while the number of G1-phase cells, and control cultures increases with time because of thymidine into DNA. The number of S-phase cells in the treated and untreated tumour cultures are compared near the end of the incubation period by incorporation of 3H-labelled thymidine into DNA. The number of S-phase cells in control cultures increases with time because of proliferation, while the number of G1-phase cells, and the removal of the biopsy; from this it is possible to estimate Tpot (9). This technique has been used for many different human tumours, providing Tpot values that vary over a range from 2 days to several weeks with a median of approximately one week. Several studies have shown that Tpot values are related to clinical outcome; at least for some tumour types high proliferation rates, corresponding to short Tpot values, are related to poor prognosis (10,11).

The above two methods of obtaining Tpot values provide estimates of cell proliferation rates in human tumours in the range 1-40% per day, with a median of about 10% per day. For a typical human tumour with a net volume doubling time of 2 months, it is relatively simple to calculate tumour cell turnover from a combination of cell proliferation rate and net growth. Thus, for such a tumour with a diameter of 2 cm, the tumour cell loss rate is about 9% per day, only slightly less than the proliferation rate of 10% per day. Thus, tumour cell loss is an important characteristic of human cancer.

4. TUMOUR CELL TURNOVER

Cell loss by tumours is much lower than that by rapidly proliferating normal tissues such as haematopoietic tissue, the gut epithelium and the skin; several million normal cells are lost from normal tissues per second, as compared to about 1000 cells per second for a 2 cm tumour. However, normal tissues have well designed systems to remove dead cells; dying epithelial cells are discharged from the skin and gut epithelium while haematopoietic cells are processed by the spleen and other specialised tissues. In contrast, most dying cells in tumours must be removed locally by phagocytes; phagocytic cells, particularly macrophages, can constitute up to 50% of the cells in tumour tissue (16). In the quoted 2 cm diameter tumour, phagocytic cells are recycling hundreds of thousands of apoptotic bodies per second. Tumour cells also die by necrosis but the efficiency with which necrotic tissue is removed by phagocytosis is low, suggesting that this is a minor pathway for tumour cell turnover. The duration of cellular apoptosis is in the order of 60 minutes, meaning that in the above tumour example, somewhat less than 1% of cells in a tumour are undergoing apoptosis at any one time, in broad agreement with experimental measurements of apoptotic index (6).

The above calculations raise two questions: why do tumours have moderately high rates of apoptosis and why are shorter Tpot values, and correspondingly increased cell turnover, often related to poor patient survival? These questions could be addressed if high rate of apoptosis were advantageous to tumour growth, and there is much experimental evidence that tumour cell apoptosis limits host immune responses to tumour (17). High rates of apoptosis in some normal tissues may also be immunosuppressive and might help to explain why a developing foetus is not rejected by the maternal immune system; it has been suggested that at an early stage of development, the blastula produces large numbers of trophoblasts that by undergoing apoptosis contribute to a state of local immunosuppression (18). Thus, rapid tumour turnover might lead to poor survival of some tumours through the effects of apoptotic cells on the stromal environment. It is worth noting that the overall rate of tumour growth, because it is lower than the rate of tumour cell division, it is likely to be controlled by factors in the tumour stromal environment such as angiogenesis and innate immunity rather than by cell division per se.

5. THE EXISTENCE OF MORE RAPIDLY GROWING CELL POPULATIONS IN HUMAN TUMOURS

As described above, tumour material removed at surgery can be cultured for a short time (7 days) and used
The paradox of cancer cell apoptosis

**Figure 2.** Relationship between calculated culture cycle times (Tpot values) of primary cultures of clinical samples and those of cell lines derived from them. Graph based on data collected in the author’s laboratory.

**Figure 3.** Theoretical model relating three tumour cell populations; a largely quiescent but self-renewing stem cell population (A), a rapidly proliferating self-renewing stem cell population (B) and a more slowly proliferating but apoptosis-susceptible cell population (C). The transition rates between each population, and from population (C) to apoptosis, are shown by the constants $k_1$, $k_2$ and $k_3$. Corresponding populations in culture are shown on the right. See text for further details.

To derive estimates of Tpot. Continued culture of such primary cultures with appropriate feeding of cells leads to death of most of the culture population. However, if culture is extended for 3 – 12 months, a more rapidly growing population of cells emerges and this can be passaged continuously as a cell line. Work in this laboratory has resulted in the establishment of more than 100 cell lines from melanoma, glioma, ovarian cancer and some other tumours (19,20,21,15). The Tpot values of many of these cell lines have been measured and found to be shorter than those of the original primary cultures. Surprisingly, there is a weak but significant relationship ($n = 73$; $r = 0.38$; $p < 0.001$) between the Tpot values of the primary culture and corresponding cell lines (Figure 2). This relationship can be used as a basis for the suggestion that cells in the more rapidly growing population, which is resistant to cell death, undergo a transition to more slowly cells which are susceptible to apoptosis. Thus, the paradox of tumour cells being both resistant and sensitive to apoptosis is resolved by postulating two populations as shown by the scheme in Figure 3. Population (B) is relatively rapidly growing and is resistant to apoptosis, while population (C) is relatively slowly growing with a high susceptibility to apoptosis. The relative proportions of populations (B) and (C) is determined by the rate constants $k_1$ and $k_3$. Such a relationship has been modelled mathematically (22).

A recent review of stem cells in normal tissue has discussed the existence of two populations of stem cells, one quiescent and one proliferating, and with each having a microenvironment that maintains its cytokinetic status (23). The scheme in Figure 3 might therefore apply largely to normal tissues with the exception that population (C) here becomes fully differentiated. This scheme implies that population (B) in normal tissues is rapidly proliferating and is capable of migrating, raising the question of whether it has properties similar to those of the corresponding tumour cell population. One of the recognised hallmarks of tumour cells is their preference for glycolysis over respiration for energy production (24). Studies with T-lymphocytes show that they have a quiescent population which, in response to immunological demand, undergoes a transition to a proliferating and migrating population that eventually matures to become differentiated memory T-cells. The proliferating population is under the control of the mTORC1 complex and relies on glycolysis rather than respiration (25). It will be interesting to determine whether proliferating, self-renewing cells from other tissues such as the skin, gut epithelium and brain have similar metabolic properties, which might equip them better for their migratory role.

6. THE EXISTENCE OF A SLOWLY GROWING TUMOUR CELL POPULATION IN HUMAN TUMOURS

The concept of a quiescent self-renewing population of stem cells that constitutes only a very small percentage of the total tissue has been well established for normal tissue (23). A considerable body of data now supports the presence of stem cells in many human cancers including those of the bladder, brain, breast, haematopoietic system, liver, ovary and stomach (26,27,28,29). It is clear at least in the case of melanoma that a proportion of this self-renewing population cycles between proliferating and quiescent populations (30). The concept that not all tumour stem cells are quiescent resolves the difficulty of how quiescent tumour stem cell population can sustain the rapid
The paradox of cancer cell apoptosis

tumour cell turnover rate of a large tumour without itself proliferating. Recent data for normal tissues such as melanocytes, colon epithelial cells and brain cells suggest that two populations of cells with self-renewal properties are located in different microenvironments, one quiescent and one proliferating (23). This concept can be applied to tumour tissue by adding a third cell population (A) to the scheme shown in Figure 3. It is thus unnecessary to postulate a high rate of transition (k) from the quiescent population (A) to the proliferating population (B), since the self-renewing capacity of population (B) is sufficient to allow generation of population (C).

An important consideration in the scheme shown in Figure 3 is not only that the three populations exist in three different microenvironments but also that their proliferative properties are controlled by their respective microenvironments. One can take normal mouse melanocytes as an example (23). The quiescent stem cell population is located near the “bulge” of the hair follicle which is called a niche, where factors such as bone morphometric protein (BMP) actively maintain a low proliferation rate combined with self-renewing capacity. Occasionally, an asymmetric cell division within this population leads to the ejection of a cell into a microenvironment that maintains its self-renewing capacity but promotes its proliferation; this is mediated for instance by members of the wnt growth factor family. The generation of this proliferating population provides the basis for amplifying, according to demand, the number of cells that migrate to the boundary of the dermis and the epidermis to differentiate into mature melanocytes.

7. The nature of dormant tumours

A recipient of a kidney transplant was reported to have developed a melanoma of donor origin even though the donor had been disease-free for 15 years following removal of a melanoma (31). Many similar examples for melanoma and other tumours have been reported, (32). The phenomenon of tumour dormancy can be considered quite easily in terms of the model in Figure 3, where the transition from a dormant tumour to an actively growing tumour is a result not of an increase in the transition rate k, but of a decrease in the transition rate k. Such a change increases the size of population (B), which in turn increases the size of population (C). In fact the model shown in Figure 3 can be applied easily both to benign tumours and to normal tissues. In normal bone marrow for instance, population (B) is relatively small but can expand in response to infection or loss of blood (33). Such signals are likely to be microenvironmental and there is evidence that they are mediated by cytokines. Thus, in response to stress, it is not the rate of proliferation of population (B) that is increased (these cells are rapidly growing in any case) but the size of population (B). It is of interest that a recent study of colorectal carcinoma concluded that the genetic changes associated with such tumours were necessary but not sufficient for tumour formation; signals in the form of hepatocyte growth factor, generated in the stromal environment by tumour-associated fibroblasts, were essential for tumour development (34).

Genetic studies have demonstrated the presence of large numbers of mutations in human tumours. Many of these are likely to be passenger mutations and play little or no role in tumour growth, but a number of genetic changes such as the acquisition of activating mutations in the \textit{PIK3CA}, \textit{MYC}, \textit{RAS} and \textit{BRAF} genes are clearly associated with tumour growth and are often referred to as “drivers” of tumour growth (35). Some genes, such as \textit{MYC} (36), \textit{RAS} (37) have the added complexity of contributing to the induction of apoptosis as well. Experience in this laboratory with a number of melanoma cell lines has shown surprisingly that the presence or absence of activating mutations in \textit{BRAF} or \textit{NRAS} are not related to changes in proliferation rate (unpublished data). In relationship to the scheme in Figure 3, the question of which of the three transitions (i.e. k, k or k) is affected by driver mutations is an interesting one. A likely candidate seems to be k since a decrease in this transition rate will lead to an increase in the size of population (B).

8. Nature of the transitions between tumour populations

It has long been known from histological studies that the transition of cells to apoptosis appears to be a stochastic one; it is an “all or nothing” event and it is clear that tumour cells do not undergo apoptosis synchronously in response to an apoptotic stimulus. Examination of the growth of cultured tumour cell populations has similarly indicated large variability in individual cell cycle times, particularly with respect to the length of G1-phase, leading to the concept of a “restriction point” G1-phase, past which the cell is committed to enter S-phase (38) and also to the hypothesis that this is a stochastic event, i.e. generated by a transition probability (39). A mathematical model for such cells incorporating transition probabilities for movement from G1-phase to S-phase, and from G2-phase to mitosis, has been used to predict the kinetic behaviour cultured cells growing in response to exposure to anticancer drug paclitaxel (40,41) and to ionising radiation (42). What is the mechanistic basis for such transitions? One possibility is that it is the resultant of multiple oscillating intracellular and intercellular signals, and that the transition occurs when the resultant increases above a threshold value. An analogy can be found in the depolarisation and firing of neurons, which is dependent on both spatial and temporal summing of incoming and internal signals. A potential basis for such oscillating signals in tumour cells is the presence of positive feedback signalling loops; such loops are known to be present for the p53 and NF-kappa B transcription factors, and a loop has been proposed to regulate the role of the E2F transcription factor in the initiation of S-phase (43).

9. Examination of the effects of antitumour therapy

One of the features of the model shown in Figure 3 is that it makes predictions about the outcome of anticancer therapies. Because the major tumour cell population (C) has a high apoptotic rate and high turnover rate, the effects of cytotoxic or targeted therapy will have
The paradox of cancer cell apoptosis

Figure 4. Effects of radiation on cell proliferation on tumour growth. Left hand panel: a melanoma cell population, corresponding to (B) in Figure 3, is assumed to have a doubling time of 1.2 days and a surviving fraction following irradiation to 9 Gy of 3%; these are typical experimental values (52). This provides a growth delay of 6.1 days. Right hand panel: the same tumour population, now with the addition of a larger, slower growing population, corresponding to (C) in Figure 3, which is susceptible to apoptosis. The transition rates were taken to mimic the growth of a clinical tumour; the growth delay is still 6.1 days but is now small in comparison to the overall tumour growth rate.

little long-term effect; it is the effect of therapy on population (B) that will be critical in terms of outcome. Since it has been argued above that cell lines are derived from population (B), the effects of therapy on cell lines can be used to increase our understanding of what happens in the whole tumour. Calculations have been made on the effect of ionising radiation at a single dose of 9 Gray (the daily radiation dose of patients receiving radiotherapy is frequently 2 Gray per day for 5 days) on a series of human melanoma cell lines (42). The surviving fraction of cells following irradiation at 9 Gray was found experimentally to vary 3% to 10%, according to the line; analysis by flow cytometry showed that radiation caused a temporary (less than 24 hour) p53-dependent arrest of the cell cycle and that this was followed in the majority of cells by long-term arrest in G2/M-phase and in a smaller proportion by cell death. When the results are applied to population (B) alone it is clear that radiation causes a significant delay in population growth, as shown by the example in Figure 4. However, when the results are applied to the total tumour cell population that includes cell death, as in the model in Figure 3, the delay in population growth is still present but is almost insignificant in terms of overall tumour growth (Figure 4). Thus, when a clinical tumour is modelled, a radiation dose that kills 97% of the driving tumour cell population has little detectable effect on the growth of the overall tumour cell population.

10. PERSPECTIVE

The picture of tumour tissue that emerges from this review is that overall tumour growth is the resultant of three tumour cell populations with different properties that exist in three correspondingly different microenvironments. A slowly growing but self-renewing tumour stem cell population is maintained by inhibitory growth factors of the TGF beta-BMP family, secreted by stromal cells forming a niche environment (26). Cells leaving this niche adopt a proliferative and migratory phenotype with a relatively rapid cell division rate but are maintained in a self-renewing state by growth factors of members of the wnt family of growth factors; these cells are modelled by cell lines, which are likewise maintained in a self-renewing state by growth factors (for instance, components of foetal bovine serum) in the culture medium. A third population of more slowly growing cells, which have lost the properties of self-renewal and gained susceptibility to apoptosis, forms the major cell population within the tumour; cells comprising this population dominate short-term primary cultures of surgical tumour material. It is clear from the model proposed in Figure 3 that the second population (B) is the driver of tumour growth; the size of this population is small in dormant tumours and relatively larger in an actively growing tumour. The growth of this second population, as illustrated in Figure 4, also has a major bearing on response to therapy. Even therapy that induces a 99.9% reduction of this population will result only in a delay of approximately two weeks in tumour, against a tumour volume doubling time of three months. Thus, the introduction of apoptosis and cell turnover into a model of tumour cell proliferation provides a clear reason for the failure of cytotoxic therapy.

The model proposed in this review explains the lack of response of tumours that are regarded as resistant to therapy but perhaps does not explain the observed clinical responses of some tumours to radiotherapy or chemotherapy. One approach in addressing this dilemma is
to propose that host-mediated mechanisms contribute to observed responses in addition to therapy-induced tumour cell killing. At a preclinical level, it is known that murine tumours can be sensitive to a given regimen of cytotoxic therapy when grown in immunopotent mice but resistant when the same tumours are grown in immunodeficient mice (44), suggesting that immune-mediated host responses are important. A study showing that a murine tumour was responsive to radiotherapy (15 Gy) when grown in the normal host mouse, but resistant to therapy in a host mouse lacking the gene for the enzyme acid sphingomyelinase (45) suggests that macrophages and/or vascular endothelial cells, which utilise this enzyme in innate immune and vascular responses, may also play a role. There are many other examples of host mechanisms potentiating the effects of cytotoxic drugs (46). The drug vadimezan (ASA404), which acts both to stimulate innate immunity and to disrupt tumour vascular endothelial function (47), potentiates the antitumour action of chemotherapy and a variety of anticancer drugs in experimental tumours (48), supporting the role of host tissues in treatment response(49). In combination with chemotherapy, vadimezan has also produced excellent partial responses in patients with non-small cell lung cancer (50). These examples provide some optimism for the future of combining cytotoxic therapy with therapies that potentiate host-mediated antitumour responses. They also provide a caveat that at least some agents currently used in therapy have a strongly immunosuppressive effect and may antagonise potential interactions. An example of this is the use of corticosteroids, which are commonly used in human cancer therapy but which antagonise the effects of vadimezan on innate immunity (51). A key challenge for the future is to develop appropriate biomarkers that will allow the monitoring of the complex interactions between tumour cells, cancer therapeutic agents and tumour microenvironmental responses.

11. ACKNOWLEDGEMENTS

Supported by the Auckland Division of the Cancer Society of New Zealand.

12. REFERENCES


The paradox of cancer cell apoptosis


27. B.B. Tysnes: Tumor-initiating and -propagating cells: cells that we would like to identify and control. Neoplasia 12, 506-515 (2010)


The paradox of cancer cell apoptosis

43. B.C. Baguley and E.S. Marshall: Do negative feedback oscillations drive variations in the length of the tumor cell division cycle? Oncol Res (2005)


Key Words: Cell Cycle; Apoptosis; Tumour Stem Cells; Microenvironment; Radiotherapy; Tumour Immunity, Review

Send correspondence to: Bruce C Baguley, Auckland Cancer Society Research Centre, Faculty of Medical and Health Sciences, The University of Auckland, Private Bag