

Monoclonal antibody therapeutics and apoptosis

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The potential for disease-specific targeting and low toxicity profiles have made monoclonal antibodies attractive therapeutic drug candidates. Antibody-mediated target cell killing is frequently associated with immune effector mechanisms such as antibody-directed cellular cytotoxicity, but they can also be induced by apoptotic processes. Antibody-directed mechanisms, including antigen crosslinking, activation of death receptors, and blockade of ligand-receptor growth or survival pathways, can elicit the induction of apoptosis in targeted cells. Depending on their mechanism of action, monoclonal antibodies can induce targeted cell-specific killing alone or can enhance target cell susceptibility to chemo- or radiotherapeutics by effecting the modulation of anti-apoptotic pathways. This review will focus on the mechanisms by which antibodies are capable of eliciting programmed cell death either directly or indirectly within tumor cells.

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Introduction

Despite significant advances in understanding the molecular aspects of cancer, conventional therapies have not changed dramatically within the past 25 years. The toxicities and adverse side effects of these therapies and the frequent emergence of resistance in recurrent tumors have led to a considerable increase in the clinical testing of monoclonal antibodies as single-agent anticancer therapies or as facilitators of tumor cell death in combination with existing therapies. Recent advances in genetic engineering to generate chimeric, humanized, and now fully human antibodies have significantly decreased antibody immunogenicity and increased antibody half-life, two factors that substantially limited the efficacy of previous rodent-derived antibody therapeutics (Boulianne *et al.*, 1984; Morrison *et al.*, 1984; Jones *et al.*, 1986; Winter and Milstein, 1991; Yarnold and Fell, 1994; Vaughan *et al.*, 1998; Carter, 2001). Enhancements in antibody screening technologies,

including the generation of human antibody phage display libraries, human immunoglobulin-producing transgenic mice, and directed affinity maturation methodologies, have further improved on the efficiency, specificity, and reactivity of monoclonal antibodies for their target antigens (Schier *et al.*, 1996; Mendez *et al.*, 1997; de Haard *et al.*, 1999; Hoogenboom and Chames, 2000; Knappik *et al.*, 2000). As a result, the isolation of high-affinity fully human monoclonal antibodies is now commonplace. Owing to their inherent specificity for a particular target antigen, monoclonal antibodies promise precise selectivity for target cells, avoiding non-reacting normal cells.

To be effective as therapeutics for cancer, antibodies must contribute directly or indirectly to the elimination of tumor cells. Fc-dependent immune effector mechanisms have been demonstrated to contribute significantly to the cytotoxic action of certain therapeutic antibodies against tumors (Ward and Ghetie, 1995; Clynes *et al.*, 2000). However, there are many examples demonstrating that antibodies can also effect target cell killing by inducing proapoptotic mechanisms or by enhancing the susceptibility of target cells to cytotoxic therapy through the modulation of antiapoptotic pathways (Trauth *et al.*, 1989; Shan *et al.*, 2000; Benini *et al.*, 2001; Liu and Fan, 2001; Reed, 2002). Apoptosis is controlled by both positive and negative factors. Accessory proapoptotic factors, such as Bax, Bad, and Smac/DIABLO or antiapoptotic factors such as Bcl-2 and inhibitor of apoptosis (IAPs) participate in the regulation of the apoptotic process at key steps (Hengartner, 2000). Evidence demonstrates that these factors, by virtue of their control over the apoptotic process, are likely responsible for the extremes in susceptibility of tumors to conventional chemo- or radiotherapy (Ekedahl *et al.*, 2002; Igney and Krammer, 2002; Sasaki *et al.*, 2003).

Currently, five monoclonal antibody therapies have been approved in the United States for the treatment of cancer and some 70 additional antibody candidates are now in clinical investigation worldwide, accounting for approximately one-quarter of the biologics now being tested (Glennie and Johnson, 2001). A subset of antibodies that have been approved or are currently in clinical trials is listed in Table 1. Therapeutic antibodies can be classified mechanistically into several groups, as shown schematically in Figure 1. There are examples of each strategy demonstrating a direct or indirect induction of apoptosis in targeted cells. First, antibodies that

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Table 1 Selected monoclonal antibodies approved or in clinical trials for oncology indications

Antibody name	Target	Type	Mode of action ^a	Developer
Erbix (cetuximab)	EGFR	Chimeric IgG ₁	Growth factor antagonist	ImClone Systems/Bristol-Myers Squibb/Merck KgA
IMC-1C11	VEGFR2	Chimeric IgG ₁	Antiangiogenesis	ImClone Systems
Avastin (bevacizumab)	VEGF	Humanized IgG ₁	Antiangiogenesis	Genentech
Herceptin (trastuzumab) ^b	ErbB2	Humanized IgG ₁	Receptor inactivation	Genentech
Rituxan (rituximab) ^b	CD20	Chimeric IgG ₁	Crosslinking	IDEC Pharmaceuticals Genentech
Campath (alemtuzumab) ^b	CD52	Humanized IgG ₁	Crosslinking	Millenium/ILEX
TRAIL-R1 mAb	TRAIL-R1	Human IgG ₁	Receptor agonist	Human Genome Sciences Cambridge Antibody Technology
Mylotarg ^b (gemtuzumab ozogamicin)	CD33	Humanized IgG ₄	Drug conjugate	Wyeth Laboratories
Zevalin ^b (ibritumomab tituxetan)	CD20	Murine IgG ₁	Radio conjugate	IDEC Pharmaceuticals
Bexxar (tositumomab)	CD20	Murine IgG _{2a}	Radio conjugate	Corixa/Glaxo Smith Kline
BL22 (RFB4(dsFV)-FE38)	CD22	Murine Fv	Toxin conjugate	National Cancer Institute

^aRefers to mechanisms relevant to the induction of apoptotic processes. ^bFDA-approved therapeutics

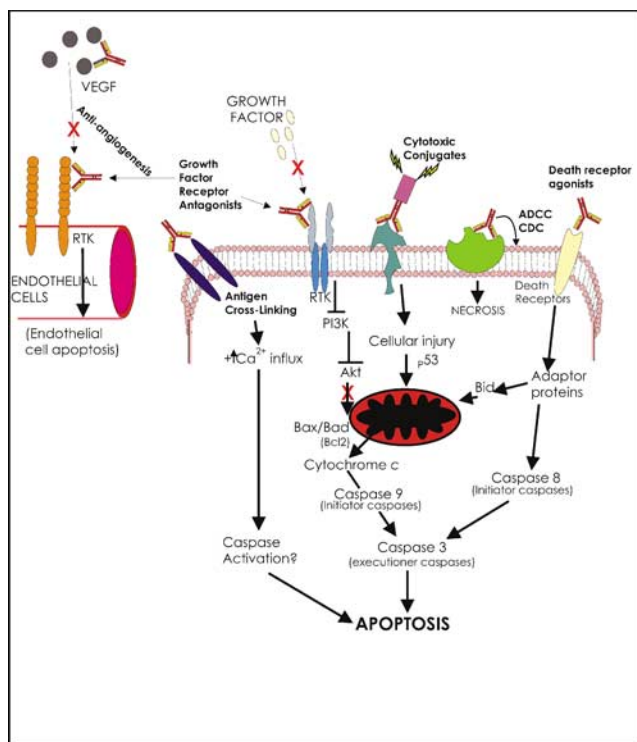


Figure 1 Antibody therapeutic strategies and the signaling mechanisms that can lead to the induction of apoptosis in targeted tumor cells

target growth factor receptors are capable of exerting a direct effect on the growth and survival of the tumor cell by antagonizing ligand–receptor signaling. As a result of receptor blockade, growth factor signaling mediated by receptor tyrosine kinase (RTK) autophosphorylation is inhibited, resulting in the arrest of tumor cell growth. In addition, because growth factor activation may also initiate antiapoptotic signaling, blocking antibodies may therefore reduce tumor cell survival mechanisms and thus enhance the efficacy of cytotoxic agents in combination therapy. Second, antibodies can be targeted to cell surface antigens and directly elicit apoptotic

signaling. Examples are antibodies that crosslink targeted surface antigen on tumor cells (Shan *et al.*, 2000; Maloney, 2001) and antibody agonists that mimic ligand-mediated activation of certain receptors (death receptors) (Trauth *et al.*, 1989; Nagata, 1997). Third, conjugated antibodies target tumor cell surface antigens and can induce localized tumor cell apoptosis by targeted delivery of cytotoxic agents. These antibodies have been chemically linked to toxic substances such as radioisotopes, bacterial toxins, or toxic chemicals (Deardon, 2002).

Some therapeutic antibodies, particularly those of human class IgG₁, can potentially direct significant target cell killing via immune effector signaling cascades' antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cellular cytotoxicity (CDC). These processes, while likely important to the biological activity of certain antibodies, represent lytic or phagocytic mechanisms for cell killing and are beyond the scope of this article. The reader is directed elsewhere for more information on this area (Ward and Ghetie, 1995; Clynes *et al.*, 2000; Presta, 2002). In the following section, we will describe in detail each particular antibody class, citing particular examples, and describing the evidence for tumor-targeted cell killing through apoptosis and its implications for anticancer therapy.

Antibodies that modulate growth and survival pathways

Both normal and tumor cells possess proliferative signaling pathways that can be triggered by exogenous growth factor signals. In normal cells, these processes are tightly controlled. On the contrary, in transformed cells, growth factor signaling mechanisms are frequently altered leading to deregulated cell proliferation. In addition, many growth factors, including epidermal growth factor receptor (EGFR), insulin-like growth factor-1 receptor (IGF-1R), fibroblast growth factor receptor (FGFR), platelet-derived growth factor receptor (PDGFR), and vascular endothelial growth factor receptor (VEGFR), stimulate not only mitogenic pathways but also activate survival pathways (Burgering and

Coffer, 1995; Parrizas *et al.*, 1997; Ong *et al.*, 2001; Danielsen and Mailhe, 2002; Harmey and Bouchier-Hayes, 2002). In particular, growth factor stimulation of the phosphoinositol 3-kinase (PI3K) pathway leads to downstream activation of the serine/threonine kinase Akt/protein kinase B (see also Kennedy *et al.*, 1997; Datta *et al.*, 1999; reviewed in Cantley, 2002). The activation of Akt induces cell survival by the inactivation of proapoptotic factors such as Bad and caspase-9. Since growth factor ligands and receptors are often overexpressed in tumors and tumor cell lines, the activation of survival processes can lead to enhanced resistance of tumors to conventional cytotoxic therapy. In fact, overexpression of a receptor or ligand in tumor cells is frequently correlated with poor clinical prognosis (Pollak, 2000; Kim *et al.*, 2001). Targeting growth factor receptors on tumor cells with antagonist antibodies may therefore inhibit key survival mechanisms, thereby promoting apoptosis and increasing cell susceptibility to cytotoxic therapy.

ErbB receptor antibodies

Therapeutic strategies to modulate growth factor signaling in tumors have been developed and have demonstrated effectiveness *in vitro* and in clinical trials. To this end, EGFR, also referred to as HER1 or ErbB-1, represents one of the most promising targets in tumor biology (reviewed in Huang and Harari, 1999; Baselga, 2002; de Bono and Rowinsky, 2002). A large body of experimental evidence supports a role for EGFR activation and signaling in the pathogenesis of a variety of human cancers. In addition, several tumor types coexpress EGFR and its ligand transforming growth factor- α (TGF- α), indicating a potential for autocrine activation. The major signaling pathways activated by EGFR include the Ras/mitogen-activated protein kinase (MAPK), phospholipase C γ , and PI3K/AKT pathways, and contribute to the control of metabolic processes, cell cycle progression, cell migration and motility, cell proliferation, apoptosis, and neovascularization (Arteaga, 2002).

The importance of EGFR in the pathogenesis of human cancer has provided the rationale for the development of EGFR antagonists as potentially useful therapeutic agents. Monoclonal antibodies that inhibit EGFR function offer a highly specific class of EGFR antagonists. To this end, a number of anti-EGFR antibodies have been developed and are in preclinical and clinical testing. One such antibody, cetuximab (IMC-C225), is a chimeric version of the murine 225 antibody developed by Mendelsohn and co-workers (Sato *et al.*, 1983; Mendelsohn, 1990). Cetuximab binds with high affinity and specificity to the extracellular domain of the human EGFR and functions as a competitive antagonist to inhibit ligand binding. As a result, cetuximab inhibits ligand-induced tyrosine kinase-dependent phosphorylation and downstream signaling of the receptor, effecting an inhibition of cell proliferation in several different human tumor lines *in vitro* and in xenograft tumor models *in vivo* (reviewed in

Mendelsohn, 1997, 2000). The DiFi human colon tumor cell line, for example, is uniquely sensitive to EGFR blockade. DiFi cells are unusual in that they express high levels of EGFR and TGF- α and are dependent upon this autocrine loop for growth and survival in culture. Antibody blockade of the EGFR pathway in DiFi cells has been shown to result in cell cycle arrest and apoptosis (Wu *et al.*, 1995; Mandal *et al.*, 1998; Liu *et al.*, 2000). Increased levels of the proapoptotic factor Bax were demonstrated in tumor cells exposed to cetuximab alone. Cell survival relies on the delicate balance between pro- and antiapoptotic factors. The lack of a functional Bcl-2 in DiFi cells likely tips the balance in favor of programmed cell death following cetuximab treatment. However, direct induction of tumor cell apoptosis involving upregulation of Bax and inactivation of Bcl-2 following cetuximab exposure has also been demonstrated in other tumor lines, including squamous cell carcinoma of the head and neck (SCCHN) and breast carcinoma (Tortora *et al.*, 1999; Huang and Harari, 2000).

The EGFR pathway is known to regulate the survival of cells through the PI3K/Akt pathway. As a likely consequence, increased EGFR expression in tumors has been associated with increased resistance to chemo- or radiotherapy in breast (Nicholson *et al.*, 1989), lung (Volm *et al.*, 1992), ovarian (Fischer-Colbrie *et al.*, 1997), and head-and-neck cancer (Sheridan *et al.*, 1997). It would be anticipated that antagonism of EGFR signaling by treatment of tumor cells with cetuximab would inhibit the activation of survival processes and enhance the effectiveness of cytotoxic agents. In fact, cetuximab has been shown to augment the antitumor activity of cytotoxic drugs and radiation therapy *in vitro* and human tumor xenograft models in mice (Baselga *et al.*, 1993; Ciardiello *et al.*, 1999; Prewett *et al.*, 1999; Tortora *et al.*, 1999; Bianco *et al.*, 2000; Bruns *et al.*, 2000; Milas *et al.*, 2000; Overholser *et al.*, 2000). In these studies, the combined effects of antibody and cytotoxic agent were markedly enhanced over treatment with either agent alone. Although several mechanisms likely contribute to this outcome, the modulation of survival processes by inhibition of the growth factor likely figures prominently. Cetuximab is currently in late-stage clinical trials investigating its activity on a variety of tumor indications as single agent therapy and in combination with conventional cytotoxic therapy.

One of the most intriguing proapoptotic mechanisms associated with cetuximab antitumor activity is its effect on DNA repair. Studies have demonstrated that inhibition of the EGFR pathway by antibody blockade with cetuximab in some tumor cell lines can suppress DNA repair mechanisms initiated following exposure to ionizing radiation (IR) and induce programmed cell death (Bandyopadhyay *et al.*, 1998; Huang *et al.*, 1999; Huang and Harari, 2000). Combination treatment of cetuximab and radiation on squamous cell carcinoma cells effected a significant enhancement in radiosensitivity and amplification of radiation-induced apoptosis. In addition to effecting increases in the level of Bax and p27^{KIP1}, a key cyclin-dependent kinase inhibitor, cetux-

imab treatment of tumor cells was shown to induce the redistribution of DNA-dependent protein kinase (DNA-PK), a critical component of DNA double-strand break repair, from the nucleus to the cytoplasm. Cetuximab treatment was shown to inhibit tumor cell recovery from potentially lethal dose radiation, suggesting that the capacity of the tumor cells to accomplish effective repair following radiation-induced DNA damage was compromised.

Another clinically relevant growth factor receptor of the ErbB family is HER2, also called ErbB2 or *neu*. This RTK is found to be overexpressed in approximately 30% of human breast carcinomas and correlates with lower survival and shorter time to relapse (Slamon *et al.*, 1987). Unlike other ErbB family members such as EGFR, a ligand has not yet been identified that binds to HER2. It can, however, form homodimers and heterodimers upon amplification or constitutive activation and can also potentiate the signaling of other ligand-binding ErbB receptors (Dougall *et al.*, 1994; Alimandi *et al.*, 1995; Penuel *et al.*, 2001).

HER2 is the target for the humanized monoclonal antibody Herceptin (trastuzumab), which binds selectively to the extracellular domain of HER2 and induces regression in receptor-positive tumors. It has been approved for the treatment of breast cancer in tumors that overexpress HER2. Evidence demonstrates that trastuzumab can participate in the induction of apoptosis by abrogation of receptor signaling of cellular survival pathways (Pietras *et al.*, 1999; Carter, 2001; Yakes *et al.*, 2002). Modulation of HER2 tyrosine kinase signaling by trastuzumab may occur by the downregulation of surface-associated receptor through internalization (Carter *et al.*, 1992). Alternatively, the antibody may inhibit heterodimerization with other ErbB receptors or ligand-dependent activation of heterodimers (Penuel *et al.*, 2001).

Treatment with trastuzumab has been shown to enhance breast tumor cell susceptibility to IR significantly. Following antibody treatment, there was a significant decrease in unscheduled DNA synthesis in response to IR exposure and a reduction in the repair of radiation-induced DNA damage. This inhibitory effect of antibody on repair was postulated to occur due to modulation in the level of p21WAF1, a mediator of cell cycle arrest following DNA damage. A similar effect was observed in tumor cells treated in combination studies of trastuzumab and taxol (Lee *et al.*, 2002). Anti-HER2 antibody therefore can modulate the HER 2 signal on tumor cells and thereby increase their susceptibility to cytotoxic agents. Preclinical and clinical results have demonstrated significant benefit when trastuzumab was used in combination with cytotoxic agents, including cisplatin, etoposide, and paclitaxel (Pegram *et al.*, 1999; Slamon *et al.*, 2001).

Targeting receptors such as the ErbB family of RTKs with antagonist monoclonal antibodies has demonstrated that inhibition of growth factor-dependent survival pathways can, at times, lead directly to an induction of apoptosis or can substantially improve the response of tumors to conventional therapy. By

blocking receptor-mediated signal transduction, these antagonistic antibodies can affect the activity of not only proapoptotic mechanisms but also DNA repair and cell cycle control, increasing the prospects for positive clinical responses in patients who may not respond significantly to cytotoxic therapy alone.

Antiangiogenic antibodies

A promising arena for antigrowth factor monoclonal antibody therapy is antiangiogenesis. Angiogenesis, the formation of nascent blood vessels, is considered essential for both primary tumor growth and metastasis (Folkman, 1991; Carmeliet and Jain, 2000). Among the numerous vascular growth factors identified, VEGF is one of the most consistently found upregulated in a number of pathological conditions associated with angiogenesis, including cancer, diabetic retinopathy, and rheumatoid arthritis (Ferrara, 1995; Folkman, 1995).

The biological activity of VEGF is mediated by two structurally related tyrosine kinase receptors, VEGFR1 (Flt-1) (Shibuya *et al.*, 1990; De Vries *et al.*, 1992) and VEGFR2 (KDR, or Flk-1 in mouse) (Terman *et al.*, 1992; Millauer *et al.*, 1993). It is generally believed that KDR is the major receptor that mediates the biological activities of VEGF, especially endothelial cell proliferation (Waltenberger *et al.*, 1994; Li *et al.*, 2000; Gille *et al.*, 2001). VEGF has also been shown to act as a potent survival factor protecting endothelial cells from apoptosis via activation of PKC or PI3K-Akt pathways and upregulation of antiapoptotic proteins such as Bcl-2, XIAP, and survivin (Gerber *et al.*, 1998a, b; Tran *et al.*, 1999; Mallat and Tedgui, 2000; O'Connor *et al.*, 2000; Mesri *et al.*, 2001; Nor *et al.*, 2001; Harmeiy and Bouchier-Hayes, 2002).

By promoting angiogenesis, especially in the hypoxic environment of tumors, VEGF functions indirectly as a potent survival factor for tumor cells (Baek *et al.*, 2000). The inhibition of VEGF signaling, either by antibody neutralization of the ligand or blockade of the receptor, has been shown to inhibit tumor growth and metastasis in a variety of animal tumor models by depriving tumors of nutrient-providing blood vessels and forcing the tumor cells into stress-induced apoptosis (Kim *et al.*, 1993; Witte *et al.*, 1998; Bruns *et al.*, 2000; Shaheen *et al.*, 2001; Rosen, 2002; Zhu *et al.*, 2002). Interestingly, recent studies have demonstrated that certain leukemia cells express functional VEGFR2 (KDR) and that VEGF can act directly as a mitogenic and survival factor for these tumor cells in a paracrine or autocrine manner (Dias *et al.*, 2000, 2001). To this end, treatment of these leukemia cells *in vitro* with the KDR antagonist antibody IMC-1C11 could inhibit cell proliferation, survival, and migration and *in vivo* significantly prolonged the survival of tumor cell-inoculated mice (Dias *et al.*, 2000, 2001). Since IMC-1C11 does not crossreact with murine Flk-1 receptor on mouse vasculature, the antitumor activity *in vivo* is most likely due to an inhibition of VEGF signaling directly on the leukemia cells. These results therefore suggest that anti-VEGF

antibody therapies have the potential to act both indirectly as antiangiogenic agents and directly as antitumor agents to inhibit tumorigenesis.

Anti-VEGF/VEGFR-based therapies have also been shown to be able to enhance the cytotoxicity of conventional chemo- and radiotherapy. Antibody DC101 has been demonstrated to potentiate the anti-tumor effects in xenograft models of several chemotherapeutic agents, including paclitaxel, cyclophosphamide, and gemcitabine (Inoue *et al.*, 2001; Bruns *et al.*, 2002), as well as of radiation (Kozin *et al.*, 2001). In addition, DC101 was shown to effect enhanced tumor growth inhibition from continuous low-dose doxorubicin treatment without additional toxicity to the host animal (Zhang *et al.*, 2002). *In vitro*, human endothelial cells, HUVEC, treated with this combination caused a significant increase in activated caspase-3 over either agent alone, with a concomitant increase in apoptosis.

Anti-VEGF ligand antibody has also been shown to enhance the susceptibility of tumor cells to IR, paclitaxel, and topotecan (Soffer *et al.*, 2001; Gupta *et al.*, 2002; Hu *et al.*, 2002). Preclinical animal studies have thus shown that blockade of VEGF signaling therefore can modulate not only endothelial cell proliferation and survival but also exert a significant effect, whether direct or indirect, on tumor cell survival and growth. Currently, the humanized form of the anti-human VEGF antibody A4.6.1, Avastin (bevacizumab), is being investigated in late-stage clinical trials in combination with chemotherapeutic agents (Jain, 2002). In addition, chimeric monoclonal antibody IMC-1C11, which targets the human KDR receptor is currently being studied in early single agent clinical trials (Posey *et al.*, 2002).

Antibodies that directly activate apoptotic signaling

Antibody-mediated antigen crosslinking

It is often considered that much of the biological activity of antibody therapeutics is attributed to the induction of natural immune effector mechanisms. However, a number of antibodies that are currently in use or in clinical testing for the treatment of hematological malignancies, including (anti-CD20) antibodies rituximab and tositumomab and (anti-CD52) antibody CAMPATH-1H, have been shown to induce apoptosis in tumor cells directly and this activity may contribute significantly to their clinical performance (Rowan *et al.*, 1998; Shan *et al.*, 2000; Cardarelli *et al.*, 2002; van der Kolk *et al.*, 2002).

Rituximab has been approved in the US as a single agent therapy for the treatment of non-Hodgkin's lymphoma (NHL), and clinical trials are currently ongoing testing rituximab in combination with conventional chemotherapy (Deardon, 2002). Tositumomab is the antibody component of Bexxar, a radioimmunoconjugate, which is currently being considered for approval for NHL (Deardon, 2002). The target for these antibodies, CD20, is expressed at high levels on

most normal and neoplastic B cells. Evidence suggests that CD20 can act as a calcium channel to initiate intracellular signals and modulate cell growth or differentiation, but its precise function is not clear (Tedder and Engel, 1994; Maloney, 2001). It has been demonstrated that upon binding to CD20, these antibodies can induce apoptosis through CD-20 dimerization (Shan *et al.*, 2000; Ghetie *et al.*, 2001; Cardarelli *et al.*, 2002). Interestingly, it was determined that the Fc portion of the antibody was dispensable for inducing apoptosis, suggesting that the immune effector functions were not necessary for this process (Cardarelli *et al.*, 2002).

The mechanism by which these antibodies can induce apoptosis may be somewhat atypical since it appears to be insensitive to Bcl-2, is not dependent on Fas, and is only partially blocked by a caspase inhibitor (Shan *et al.*, 2000; van der Kolk *et al.*, 2002). It has been proposed that increases in intracellular Ca²⁺ levels, which occur in response to anti-CD20 crosslinking, may be involved in the apoptotic process, but the precise mechanism remains to be elucidated. Activated caspase-3 and caspase-9 have been detected in clinical samples from patients with chronic lymphocytic leukemia following treatment with rituximab, demonstrating that the proapoptotic effect can be detected *in vivo* in tumor cells and suggests that the mitochondria-dependent (cytochrome *c* release) pathway is at least partially involved in rituximab-dependent tumor cell death (Byrd *et al.*, 2002). Not surprisingly, in this study, the proapoptotic response to rituximab correlated with a favorable clinical outcome. To this end, the ability of anti-CD20 antibody therapy to activate proapoptotic processes, even in Bcl-2-positive cells (Miyashita and Reed, 1993; Maung *et al.*, 1994), is a likely reason why good clinical responses have been observed in both single agent and combination therapy strategies.

Death receptor agonist antibodies

A series of cell surface receptors have been identified in recent years, which upon activation with ligand, can each transduce a proapoptotic signal in normal and transformed cells. The tumor necrosis factor (TNF) receptor (TNFR) family of death receptors includes TNFR1, TRAMP, TNF-related apoptosis-inducing ligand receptor (TRAIL)-R1, and TRAIL-R2 (Nagata and Golstein, 1995; Ashkenazi and Dixit, 1998). These receptors each carry a cytoplasmic region, the death domain that is necessary for transmission of the apoptotic signal following receptor activation. The death receptor machinery is vitally important to the immune system. It serves as a means for selective killing in such processes as elimination of activated T cells following an immune response and in the cytotoxic T-cell response to virally infected cells (Ashkenazi and Dixit, 1998). The notion of targeting these receptors on tumors with monoclonal antibodies to induce apoptosis is attractive since they are frequently expressed on tumor cells and can act directly to trigger apoptosis.

Trauth *et al.* (1989) described the identification of an antibody anti-APO-1 that selectively bound to the surface of a human B-cell lymphoblastoma cell line, resulting in growth inhibition and rapid induction of apoptosis. This antibody bound to a 48 kDa protein designated APO-1 and later identified as Fas. The anti-APO-1 antibody acted as a ligand mimic, stimulating the signaling of the death receptor. *In vivo*, a single 500 μ g injection of antibody into a nude mouse xenograft model induced rapid regression of large tumors and normal tissue showed no adverse effects. Others, however, have demonstrated that anti-Fas receptor agonist antibodies can effect significant hepatotoxicity *in vivo* (Ogasawara *et al.*, 1993), and it has since been determined that the Fas receptor is expressed in a wide variety of normal tissues, including the thymus, liver, heart, and kidney (Nagata, 1997).

More recently, a mouse monoclonal antibody, TRA-8, has been developed as an agonist of the TRAIL-R2 (DR5) receptor and shown to induce selective apoptosis in a variety of tumor cells *in vitro* and *in vivo* (Ichikawa *et al.*, 2001). TRA-8 has been shown to be inhibitory to both solid tumors and leukemic cell lines in animal models. In addition, when used in combination with a protein kinase C inhibitor, TRA-8 synergistically enhanced the level of apoptosis in tumor cells (Ohtsuka and Zhou, 2002). TRA-8 did not exhibit any cytotoxicity to normal hepatocytes *in vitro*, suggesting that targeting TRAIL-R2 with agonist antibodies may selectively kill tumor cells and prevent damage to normal cells (Ichikawa *et al.*, 2001).

Resistance to TRAIL-induced apoptosis has been demonstrated in tumor models and may be problematic in the clinical application of death receptor therapy (Trauth *et al.*, 1989; Scaffidi *et al.*, 1998; Findley and Zhou, 1999). For example, activation of the PI3K/Akt survival pathway, upregulation of Bcl or Bcl-xL, or expression of the adaptor protein blocker FLIP can block TRAIL-mediated apoptosis in tumor cells (Irmeler *et al.*, 1997; Scaffidi *et al.*, 1998; Kandasamy and Srivastava, 2002). Interestingly, since Bcl2 overexpression is a frequent occurrence in tumors, the combination of TRAIL agonists with monoclonal antibodies that block survival pathway signaling may be an effective antitumor therapy.

Recently, two nonsignaling TRAIL-binding receptors, TRAIL-R3/DcR1 and TRAIL-R4/DcR2, were identified that are found predominantly on normal cells (Pan *et al.*, 1997, 1998). These so-called decoy receptors may in part be responsible for the selective resistance of normal cells to TRAIL-induced apoptosis (Baetu and Hiscott, 2002). It may therefore be advantageous when screening for effective TRAIL agonist antibodies to identify selective binding to TRAIL-R1 or -R2, as was accomplished with TRA-8.

Although it remains to be seen whether death receptor agonist antibodies are clinically useful as anticancer therapeutics, preclinical results demonstrate that this class of antibodies can direct tumor-specific cell killing by initiating apoptotic signaling and may provide greater versatility in target selection over the therapeutic

use of the death receptor ligand. Clinical trials have recently been initiated to test a fully human TRAIL receptor agonist antibody (TRAIL-R1 mAb) in patients with advanced forms of cancer.

Antibodies that target the delivery of cytotoxic compounds

An approach to maximize the efficacy of monoclonal antibodies involves the conjugation of antibodies or their subunits to a variety of cytotoxic agents. The principal rationale behind this immunoconjugate approach is that it takes advantage of the specificity offered by antibodies to deliver cytotoxic agents directly, and in higher local concentrations, to tumor tissues while avoiding damage to normal cells. Higher effective concentrations of drug or radiation at the tumor site would then be anticipated to increase the extent of localized cellular damage and, as a result, enhanced tumor cell apoptosis. Agents currently conjugated to antibodies include radioisotopes, toxins, enzymes/prodrugs, and cytokines.

Radio-immunoconjugates

IR is an effective anticancer cytotoxic agent. Radio-immunoconjugate antibodies have the potential to combine the effectiveness of radiation with the specificity of a monoclonal antibody directed to a tumor-specific target. Hematopoietic malignancies are particularly sensitive to radiation and often carry many well-characterized and selectively expressed cell surface antigens that make excellent candidates for antibody targets. To this end, hematopoietic cancers have been frequently selected as targets for radioconjugates. The therapeutic radioisotopes of choice have been the beta-emitters – yttrium-90 and iodine-131. Human clinical trials of these isotopes as conjugates to anti-CD20 antibodies in the treatment of NHL have been successful. Bexxar (tositumomab) is an I-131 conjugate awaiting FDA approval, while Zevalin (ibritumomab tituxetan), a Y-90 conjugate, was approved in 2001 (Witzig *et al.*, 1999; Press *et al.*, 2000). To highlight the enhanced effectiveness of radioimmunoconjugates, radiolabeled anti-CD20 monoclonal antibody Zevalin was compared to rituximab in a randomized, controlled, multicenter study involving 143 low-grade NHL patients (Witzig *et al.*, 1999, 2002). The Zevalin group benefited from higher overall response rates, 80 versus 44%. Moreover, the treatment was well tolerated. Tumor susceptibility to IR is dependent on multiple factors, in particular p53 status and Bcl2 overexpression (Peltenburg, 2000). With this in mind, it is possible that direct cellular effects of the antibody itself, such as Bcl-2-independent apoptosis induced by CD-20 crosslinking with tositumomab, may elicit a combinatorial effect with the localized radiation, providing enhanced tumor cytotoxicity. In addition, conjugate therapy facilitates localization of the IR to the site of the tumor. High-energy beta-emitters can then kill adjacent

antigen-negative tumors cells, increasing overall toxicity to the tumor.

Toxin-immunoconjugates

As an alternate strategy to radioimmunoconjugates, toxins from bacteria (e.g. diphtheria toxin or *Pseudomonas* exotoxin (PE)) or plants (e.g. castor bean-derived ricin or gelonin) have been most commonly used for antibody conjugation (For a review of a wide variety of immunotoxins, see Kreitman, 2001). The rationale for their use is based on their extreme potency as cytotoxic agents; a single molecule can kill a cell. In this approach, the cell surface antigen must be one that internalizes upon antibody binding, thereby introducing the toxin to the cell's interior where it is released and activated to interfere typically with protein synthesis and induce apoptosis. Mylotarg, approved by the FDA in 1999 for the treatment of refractory acute myelogenous leukemia, is an anti-CD33 antibody conjugated to calicheamicin and serves as the precedent for this approach (Sievers *et al.*, 1999; Sievers, 2000). Once internalized, calicheamicin induces apoptosis by binding DNA and causing strand breaks. Other immunotoxins are usually made up of the variable domains of an antibody (Fv) fused to a truncated recombinant toxin protein. An example is BL22 (RFB4(dsFV)-PE38), which is currently under evaluation in human clinical trials of NHL and other leukemias (Kreitman *et al.*, 1999, 2000; Kreitman, 2001). The antibody portion of BL22 is conjugated to truncated PE and targets CD22, a B-cell-restricted cell adhesion molecule. Following internalization of the BL22 complex to the cytosol, the ADP ribosylating domain of PE separates from the dsFV antibody subunit and inhibits elongation factor 2 and protein synthesis. Tumor cell killing is induced as a result of inhibition of protein synthesis in a caspase-dependent process (Keppler-Hafkemeyer *et al.*, 2000). Cell lines pretreated with peptidyl caspase inhibitors were shown to be 2–5-fold more resistant to BL22. A major limitation to the use of protein–antibody conjugates in cancer patients is the frequent formation of an immune response to the foreign toxin protein that subsequently compromises its biological activity.

Antibody-directed enzyme prodrug therapy (ADEPT)

In this approach, an enzyme is localized to a non-internalizing cell surface tumor antigen by virtue of its conjugation to a monoclonal antibody. Once the excess antibody–enzyme conjugate has had the opportunity to clear from systemic circulation, an antitumor prodrug is administered. The enzyme then rapidly converts the prodrug into its active form directly at the tumor site. As an alternative strategy to conventional drug delivery, ADEPT theoretically allows the targeting of chemotherapeutic agents to tumor masses while preventing nonspecific toxicities. In the past few years, several enzyme–prodrug combinations have demonstrated success in preclinical models of human cancer (for a review,

see Senter and Springer, 2001). One ADEPT approach, A5B7-Fab'2-CPG2, activates a nitrogen mustard pro-drug, ZD2767P, by the bacterial enzyme carboxypeptidase G2 (CPG2) that is targeted to the tumor cell by conjugation to the Fab fragment of the anti-CEA antibody A5B7. In preclinical studies, apoptotic cell death was apparent in LoVo tumor cells following prodrug treatment of ZD2767P + CPG2 at levels comparable to the established nitrogen mustard chlorambucil (Monks *et al.*, 2001). This ADEPT strategy has also shown biological activity in Phase I trials (Bagshawe and Begent, 1996; Napier *et al.*, 2000).

Conclusion

Mechanistically, we have described four processes for the induction of apoptosis by therapeutic antibodies: (1) modulation of RTK antiapoptotic signaling, (2) antigen crosslinking, (3) death receptor activation, and (4) apoptosis in response to cytotoxic agent delivery. Significant preclinical and clinical results exist that are supportive of each strategy in the elimination of tumors through proapoptotic processes, either directly or indirectly. To this end, there are representatives of each type within the group of approved oncology antibody therapeutics, with the exception of death receptor activation, a relatively recent addition to the field, but one that holds significant promise. The data demonstrating that monoclonal antibodies can effect tumor cell killing through apoptotic processes beyond immune effector functions illustrates the versatility of this therapeutic approach and only enhances their attractiveness as anticancer agents. Utilized alone or in combination with conventional treatment, antibody therapeutics with proapoptotic potential may help to overcome the frequent problem of resistance in recurrent tumors to cytotoxic therapies by directly inducing apoptosis or by lowering the apoptotic threshold and increasing specific tumor cytotoxicity.

Abbreviations

ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cellular cytotoxicity; IAP, inhibitor of apoptosis; RTK, receptor tyrosine kinase; TNFR, tumor necrosis factor receptor; TRAIL, TNF-related apoptosis-inducing ligand receptor; EGFR, epidermal growth factor receptor; IGF-1R, insulin-like growth factor-1 receptor; FGFR, fibroblast growth factor receptor; PDGFR, platelet-derived growth factor receptor; VEGF, vascular endothelial growth factor; PI3K, phosphoinositol 3-kinase; PLC γ , phospholipase C gamma; MAPK, mitogen-activated protein kinase; TGF- α , transforming growth factor- α ; IR, ionizing radiation; SCCHN, squamous cell carcinoma of the head and neck; DNA-PK, DNA-dependent protein kinase; JNK, c-jun N-terminal protein kinase; NHL, non-Hodgkin's lymphoma; AML, acute myelogenous leukemia; CLL, chronic lymphocytic leukemia; ADEPT, antibody-directed enzyme prodrug therapy.

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