9
Cell-based Delivery Systems for Antiangiogenic Therapy

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Introduction

Angiogenesis, the growth of new blood vessels from pre-existing ones, is a complex process with multiple, sequential, and interdependent steps (Folkman, 1992; Folkman and Shing, 1992; Folkman and D'Amore, 1996). Under normal conditions, such as wound healing, the angiogenic process is tightly regulated by both stimulatory and inhibitory factors (Hanahan and Folkman, 1996). However, under certain pathological conditions, such as rheumatoid, arthritis, diabetic retinopathy, and the growth of solid tumours, angiogenesis is not under such tight regulation (van Hinsbergh *et al.*, 1999).

The hypothesis that angiogenesis is a requirement for the growth and metastasis of solid tumours was proposed nearly 30 years ago by Judah Folkman (Folkman, 1971). He hypothesized that solid tumours could not grow beyond 1–2 mm without developing their own blood supply. This observation has led to the development of a new therapeutic pathway. Angiogenesis inhibitors, which may be natural or synthetic, include tissue inhibitors of matrix metalloproteinases (Murray *et al.*, 1996; Brooks *et al.*, 1998; Bello *et al.*, 2001), chemokines (Maione *et al.*, 1990; Arenberg *et al.*, 1997; Jouan *et al.*, 1999), tyrosine kinase inhibitors (Kim *et al.*, 1993; Strawn *et al.*, 1996; Dias *et al.*, 2001; Lu *et al.*, 2001), interleukins (Luca *et al.*, 1997; Stearns *et al.*, 1999), and naturally occurring proteolytic fragments of large precursor molecules, such as endostatin, vasostatin, canstatin, angiogstatin, and others (O'Reilly *et al.*, 1994, 1997; Yamaguchi *et al.*, 1999; Kamphaus *et al.*, 2000; Lee *et al.*, 2000; Lange-Asschrnfeldt

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Abbreviations: BCE, bovine capillary endothelial cells; BHK, baby hamster kidney cells; PLA, poly lactic acid; PLL, poly-L(lysine).

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et al., 2001; Xiao *et al.*, 2002). These antiangiogenic molecules exert their inhibitory functions by multiple mechanisms on endothelial cell proliferation, migration, protease activity, as well as the induction of apoptosis. To date, more than 30 angiogenic inhibitors are in clinical trials, and many new ones are being studied *in vitro* and *in vivo* in animal models (for review, see Hagedorn and Bikfalvi, 2000).

Animal experiments and human clinical trials have shown that high and steady state levels of the antiangiogenic protein must be administrated in order to achieve and maintain antitumour effects (O'Reilly *et al.*, 1994, 1997; Jain *et al.*, 1997; Dawson *et al.*, 1999; Kisker *et al.*, 2001; Peroulis *et al.*, 2002). Due to their relatively short half-lives, antiangiogenic inhibitors have to be administrated on a long-term basis and in large, constant quantities, in order to maintain tumour inhibition. These obstacles are further compounded when administering antiangiogenic therapy for brain tumours. The ability to deliver therapeutics systemically to the brain is hampered by the presence of the blood–brain barrier, a continuous, tight-junctioned cerebral capillary endothelium. Therefore, alternative strategies and modalities need to be developed to achieve effective local and systemic therapy for brain tumours. In this review, we will discuss cell-based delivery systems for antiangiogenic therapy.

Gene therapy

Gene therapy is one way to deliver antiangiogenic proteins. It refers to the transfer of genetic material into mammalian cells with the aim of elucidating a therapeutic response. Introduction of a gene into cells can take place *ex vivo* by transfecting donor or autologous cells that have been engineered *in vitro* and then transplanted to the recipient. Genes may also be transferred directly into target cells and organs *in vivo*. Both transfection strategies are carried out with the aid of vectors, such as viruses (retroviruses and adenoviruses), liposomes, chemical agents, electroporation, and direct injection of naked plasmids (Mulligan, 1993; Nabel *et al.*, 1993; Yla-Herttuala and Martin, 2000; Meyer and Finer, 2001; Liu and Huang, 2002).

Gene therapy using angiogenic inhibitors is one approach being used to bring antiangiogenic therapy into the clinic. To date, there are nearly 200 gene therapy clinical trials for the treatment of various diseases, and more than 50% of these are designed for the treatment of cancer (Folkman, 1998; Ding *et al.*, 2001; Kuo *et al.*, 2001a; Regulie *et al.*, 2001). Antiangiogenic *in vivo* gene therapy studies have demonstrated the effectiveness of this strategy by demonstrating a reduction in tumour growth in animal models (Folkman, 1998; Lin *et al.*, 1998; Kuo *et al.*, 2001b; Regulie *et al.*, 2001). Unlike direct protein therapy, antiangiogenic gene therapy does not require high doses of DNA for injection. Therefore, the use of the gene therapy approach for antiangiogenic therapy can avoid repeated injections, and the high cost of purified protein production.

However, clinical trials using gene therapy have failed to reach phase III due to several hurdles, particularly concerning the type of viruses used (Folkman, 1998; Puumalainen *et al.*, 1998; Harsh *et al.*, 2000). Retrovirus can integrate into the cellular genome and inactivate host tumour suppression gene, or activate protooncogenes *in vivo*. Retrovirus can also promote gene transfection of a limited size gene (7 kB) and

infect only dividing cells, whereas most mammalian tissues consist primarily of non-dividing cells. Adenovirus, the second most commonly used viral vector, has the disadvantage of infecting all tissues, including germ cells, when delivered *in vivo* (Temin, 1990; Ferber, 2001). Additional challenges for *in vivo* antiangiogenic gene therapy are to understand how angiogenic inhibitors function, how tumour vessels differ from normal blood vessels, and how to target tumour vessels with appropriate therapies.

Genetically engineered cells

An alternative strategy for direct gene delivery is to deliver genetically engineered cells that overexpress the protein of interest. Cell delivery can be considered 'a living drug delivery system', which provides an unlimited protein source. As long as the cells are viable, functional, and able to sense signals from the body, they are able to release the desired products in a physiological manner. This therapeutic mode eliminates most of the complex preparation/formulation processes used in the more traditional protein/peptide delivery systems. Additionally, transplanted cells may be able to replace an entire cell type, acting as an artificial organ and providing needed metabolic functions. Genetically modified cells have been used to secrete a wide therapy of transgene products, including factor IX (Dai *et al.*, 1992; Hortelano *et al.*, 1996; Snyder *et al.*, 1997), growth hormone (Peirone *et al.*, 1998), erythropoeitin (Rinsch *et al.*, 1997), cilliary neurotrophic factor (CNTF) (Aebischer *et al.*, 1996a), dopamine (Pallini *et al.*, 1997), endostatin (Joki *et al.*, 2001), and Neuropilin-1 (Schuch *et al.*, 2002).

In general, two approaches have been used to deliver cells for therapeutic purposes. The simplest involves the direct injection of a bolus of cells in the form of a cell suspension, either into the blood stream, a body cavity, or directly into the parenchyma of a particular tissue. Alternatively, the cells may be implanted in association with one or more biomaterials that serve as a vehicle for their delivery. The biomaterial may consist of a simple extracellular matrix, or may be made of synthetic materials, or a combination of the two. This approach has been used extensively in the field of tissue engineering, where autologous or allogeneic cells are seeded onto a polymeric scaffold and then implanted *in vivo* for the replacement or regeneration of organs (Cima *et al.*, 1991; Langer and Vacanti, 1993; Kaushal *et al.*, 2001; Patrick *et al.*, 2001; Hirschi *et al.*, 2002). Among the natural polymers used for such applications are the collagens, glycosaminoglycan, starch, chitin, chitosan, and alginate (Shapiro and Cohen, 1997; Kaushal *et al.*, 2001; Shofeng *et al.*, 2001). These have been used to repair nerves, skin, cartilage, liver, bone, and blood vessels.

Many synthetic restorable polymers, such as poly(a-hydroxy ester)s, poly-anhydrides, polyorthoesters, and polyphosphazens, have been developed also for cell transplantation. By far, the family of PLA (poly lactic acid) is the most commonly used synthetic biomaterial for cell scaffolding. However, the major challenge when delivering allogenic cells (whether engineered or not) by injection or by a polymeric scaffold, is the need to circumvent the host immune reaction to the implanted cells. The seeded cells are implanted in an opened matrix, and are exposed to the host immune system.

Cell encapsulation

The encapsulation system consists of viable cells surrounded by a non-degradable, selectively permeable barrier that physically isolates the transplanted cells from host tissue and the immune system. This cell bioreactor relies on host homeostatic mechanisms for the control of pH, metabolic waste removal, electrolytes, and nutrients. Numerous encapsulation techniques have been developed over the years and classified as either microencapsulation (0.2–1.5 mm) and macroencapsulation (involving larger flat-sheet and hollow-fibre membranes). Macroencapsulation was initially developed in the 1940s by Algire (Algire, 1943), who implanted cells in a diffusion chamber. By the mid 1970s, the diffusion chamber technique had been established as a unique *in vivo* method for studying cell growth and differentiation (Weinberg and Stohlman, 1976; Ben-Ishay and Sharon, 1977). However, the disadvantages of these macrocapsules derive from their physical properties: and in particular their surface-to-volume ratio, which is unfavourable for the entrapment of cells, and leads to a high risk of membrane breakage and low cell loading capacity (Lacy, 1995; Lanza *et al.*, 1996). By contrast, microcapsules have several advantages in comparison to macrocapsules. Microcapsules have a large surface-to-volume ratio due to their relative small size. They can be prepared from a variety of biocompatible polymers with good mechanical stability, and can be easily administrated and retrieved.

One of the most promising cell microencapsulation methods has been based upon alginate. Alginates are polysaccharides extracted from various species of brown algae (seaweed) and purified to a white powder. The alginates have different characteristics of viscosity and reactivity, based on the specific algal source and the ions in solution. The alginates are linear, unbranched polymers containing α(1–4)-linked D-mannuronic acid (**M**) and α(1–4)-linked L-guluronic acid (**G**) residues. Although these residues are epimers (D-mannuronic acid residues being enzymatically converted to L-guluronic after polymerization) and only differ at C5, they possess very different conformations; D-mannuronic acid being $^4C_1$ with diequatorial links between them, and L-guluronic acid being $^1C_4$ with diaxial links between them. Hydrocolloids such as alginate can play a significant role in the design of a controlled-release product. At low pH hydration, alginic acid leads to the formation of a high-viscosity 'acid gel'. Alginate is also easily gelled in the presence of a divalent cation as the calcium ion. The ability of alginate to form two types of gel depends on pH, i.e. an acid gel and an ionotropic gel gives the polymer unique properties, compared to neutral macromolecules. So far, more than 200 different alginate grades and a number of alginate salts have been manufactured. The potential use of the various qualities as pharmaceutical agents has not been evaluated fully, but alginate is likely to make an important contribution in the development of polymeric delivery systems.

Using a hydrogel such as alginate has several advantages. The mechanical irritation to the surrounding tissue is reduced by the soft and pliable features of the gel. Second, alginate has hydrophilic properties, which minimizes protein absorbance and cell adhesion, thus exhibiting a high degree of biocompatibility. The complexation between the polyanionic alginate and a polycation poly-L(lysine) (PLL) has been the first utilized for cell encapsulation (Lim and Sun, 1980). This complex forms a semipermeable membrane, which allows the controlled delivery of different bioactive

substances *in vivo*, while preventing the diffusion of antibodies and other components of the immune system. The alginate–poly-L(lysine) membranes allow the free exchange of nutrients and oxygen between the implanted cells and the host, while preventing the escape and elimination of encapsulated cells. Alginate microcapsules have been used for various applications, particularly for the encapsulation of pancreatic islet cells for insulin delivery (Weber *et al.*, 1990; Soon-Shiong *et al.*, 1993; Siebers *et al.*, 1998). A large number of studies have shown that intraperitoneal xenograft of alginate–PLL encapsulated rat, dog, pig, or human islets into diabetic mice, dogs, or humans can regulate blood glucose levels. This method has also been used for the encapsulation of cells which release cytokines and hormones (Savelkoul *et al.*, 1994; Parkash and Chang, 1996; Thorsen *et al.*, 2000). Broad application of encapsulation cell delivery has also been demonstrated for the delivery of neuroactive agents in the treatment of different conditions, such as age-related degeneration (Emerich *et al.*, 1994, 1996), Alzheimer's disease (Hoffman *et al.*, 1993; Winn *et al.*, 1994; Emerich *et al.*, 1997b), amyotrophic lateral sclerosis (Aebischer *et al.*, 1996b), neuroprotection (Deglon *et al.*, 1996), Huntington's disease (Hammang *et al.*, 1995), and Parkinson's disease (Tresco, 1994; Emerich *et al.*, 1997a; Lindner *et al.*, 1997).

Cell encapsulation for antiangiogenic tumour therapy

Encapsulation of non-autologous cells, which are genetically engineered to secrete antiangiogenic proteins, is a unique strategy that can be used to combat tumours. Such a system facilitates the continuous release of biologically active antiangiogenic protein, thus overcoming obstacles such as the short half-life of the proteins, long-term administration, high doses, and cost. Two reports, one coming from our laboratory and one from the group of Read and co-workers, represent the first application of the encapsulation technology to angiogenesis inhibition (Joki *et al.*, 2001; Read *et al.*, 2001). Our study has demonstrated the use of alginate encapsulated baby hamster kidney cells (BHK-21), which have been engineered to continuously secrete high levels of human endostatin for the treatment of a malignant human brain tumour, glioblastoma (*Figure 9.1*). Glioblastomas are poorly understood lethal tumours with a 12–18 month median survival, despite aggressive treatment. Mitosis, invasion, and angiogenesis are three cardinal features of their behaviour. There are presently no therapeutic agents in the clinic that successfully block any one of these attributes effectively. Endostatin, a 20-kd fragment of collagen XVIII with demonstrated antiangiogenic activity, is being tested in phase I clinical trials as a protein infusion for various cancers (Eder *et al.*, 2002; Herbst *et al.*, 2002). The recombinant, biologically active protein is difficult to produce, and is rapidly cleared from the blood. Endostatin has been tested in murine tumour models, with varying success, by gene therapy delivery vectors, including adenoviral vectors, adeno-associated viral vectors, *in vitro* transfections, polymerized plasmids, and DNA cationic liposomes (Sauter *et al.*, 2000; Ding *et al.*, 2001; Kuo *et al.*, 2001b; Szary and Szala, 2001). To achieve significant tumour regression, 2.5 mg/kg recombinant endostatin was administrated once daily for 16 days in a Lewis lung carcinoma model (O'Reilly *et al.*, 1997). The quantities of protein needed for this therapy, the purification procedure for large-scale production, and the attendant costs of these processes have greatly hindered current efforts to improve its efficacy *in vivo*.

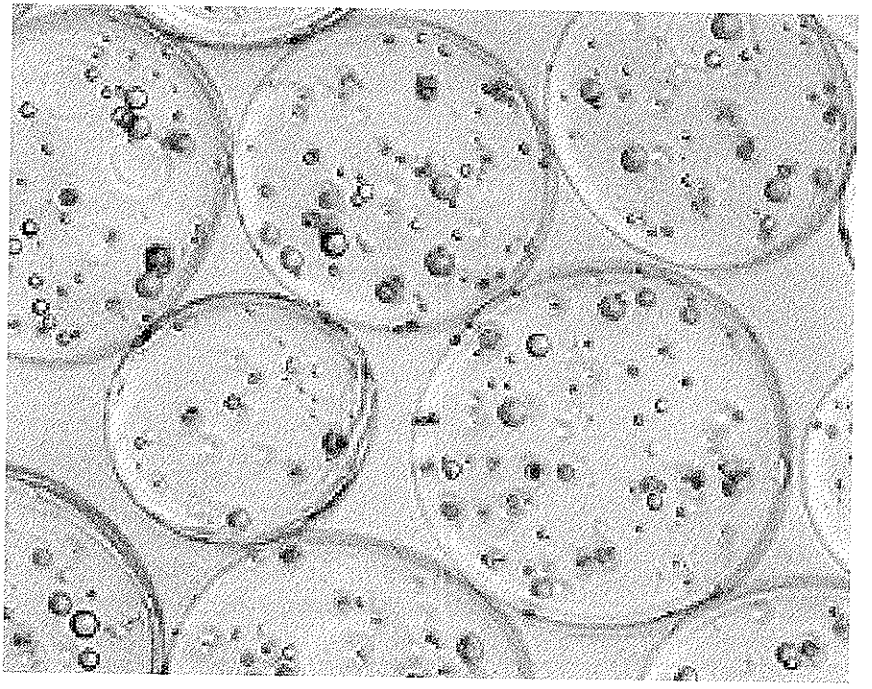

Figure 9.1. Phase-contrast microscopy of cell-loaded microcapsules shows the uniform size and cell distribution. (Reproduced with permission from *Nature Biotechnology*.)

Therefore, it seemed logical to investigate the ability of an endostatin encapsulated system for inhibiting the growth of a human glioblastoma. An *in vitro* proliferation assay performed on bovine capillary endothelial cells (BCE cells) using encapsulated baby hamster kidney (BHK)-endo cells resulted in 67.2% inhibition in proliferation over a three-day time period (*Figure 9.2*). By comparison, previous proliferation experiments had shown a 25% inhibition when the same amount of endostatin was added daily to cultured cells (O'Reilly *et al.*, 1997). The potent inhibition of BCE proliferation may be explained by the continuous release of endostatin during the course of the experiment, rather than being added exogenously once at the beginning of the assay. A semi *in vivo* tube formation assay using porcine aortic endothelial cells transfected with KDR (PAE/KDR) was employed to confirm the biological activity of the endostatin released from the capsules. Conditioned media from U-87 cells stimulated tube formation (10 tubes/well), indicating the potential antigenic activity of factors released from U-87 cells. The antiangiogenic activity of the released endostatin was confirmed by the observation that no tube formation was detected in the cells treated with U-87MG media. A series of *in vivo* studies performed in nude mice bearing subcutaneous, human glioma tumours showed that a single administration of microencapsulated-engineered BHK cells (adjacent to the tumour), which continuously secrete biologically active endostatin, significantly inhibited the tumours. Animals treated with encapsulated BHK-endo cells exhibit a 72% inhibition in tumour growth 21 days post-microcapsule injection (*Figure 9.3*). The same amount of

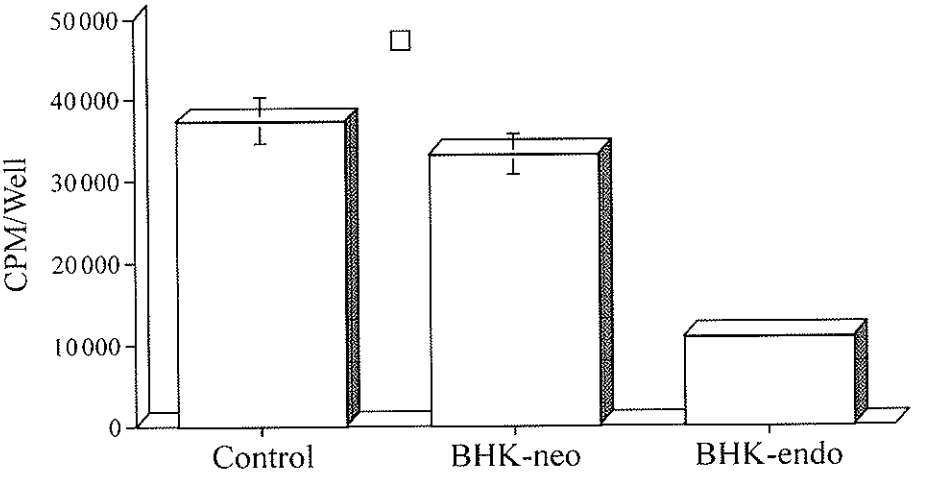

Figure 9.2. The effect of BHK-producing endostatin and BHK-neo (control) cells on the proliferation of BCE cells in culture. (Reproduced with permission from *Nature Biotechnology*.)

the implanted encapsulated BHK-endo cells has been shown to release an average of 150.8 ng/ml/week *in vitro*, which may give a indication for the concentration of endostatin released *in vivo*.

Recently, several other experimental studies using cell encapsulation for antiangiogenic therapy have been published (Orive *et al.*, 2001; Cirone *et al.*, 2002). Orive *et al.* immobilized VE-cadherin-secreting hybridoma cells in alginate–agarose microcapsules. The alginate and agarose solid beads were coated with poly-L(lysine) and the 1B5 hybridoma cells were grown within the microcapsules for 9 days of culture, reaching a cumulative concentration of 1.7 g/mL. According to the author, this antibody concentration inhibited microtubule formation (87%) in the *in vitro* angiogenesis Matrigel assay. In another study, myoblasts that had been genetically modified to secrete interleukin-2, linked to the Fv region of a humanized antibody with affinity to HER-2/neu, were encapsulated in alginate–poly-L(lysine)–alginate microcapsules (Cirone *et al.*, 2002). The efficacy of this system was tested in a mouse model bearing HER-2/neu-positive tumours. The treatment led to a delay in tumour progression, and prolonged survival of the animals. However, the long-term efficacy was limited by an inflammatory reaction against the implanted microcapsules, probably because of the secreted cytokine and antigenic response against the xenogenic fusion protein itself. Over the short-term treatment (initial 2 weeks), efficacy was confirmed when a significant amount of biologically active interleukin-2 was detected systemically, and targeting of the fusion protein to the HER-2/neu-expressing tumour was shown by immunohistochemistry. The tumour suppression in the treated animals was associated with increased apoptosis and necrosis in the tumour tissue, thus demonstrating successful targeting of the anti-proliferative effect to the tumours by this delivery paradigm.

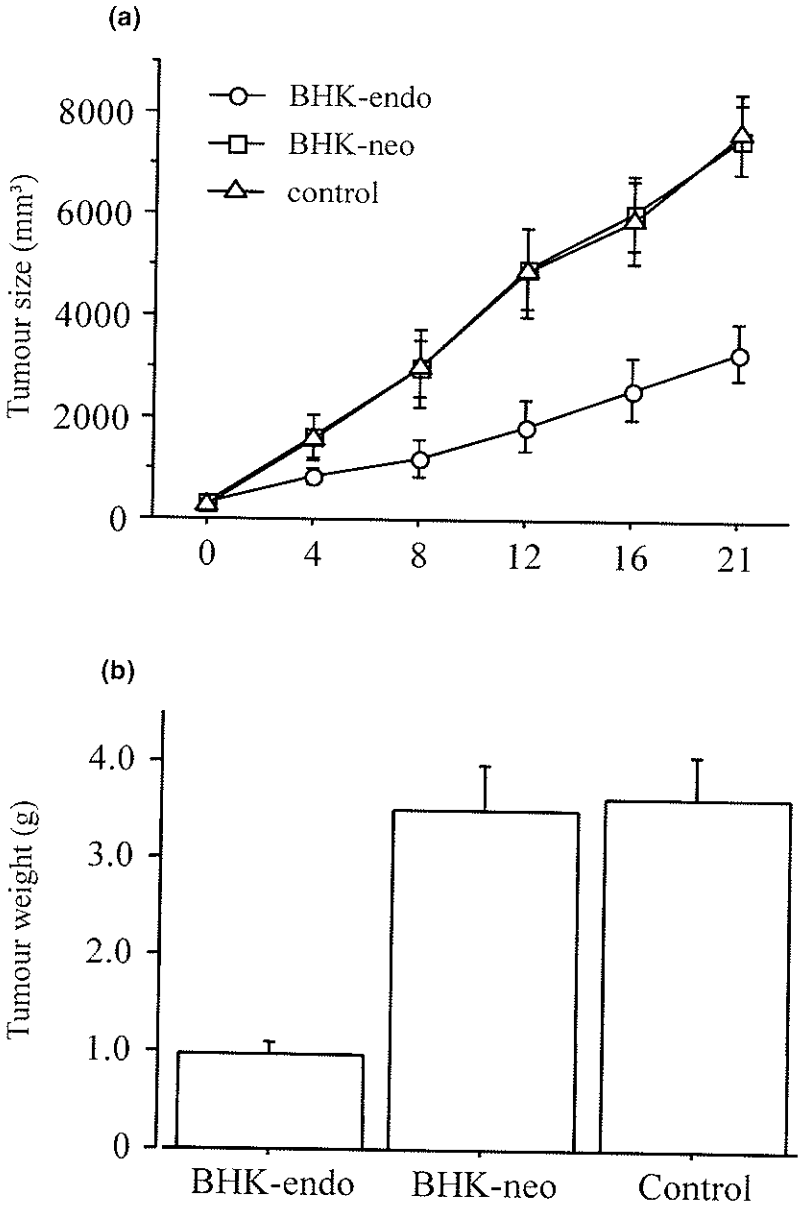

Figure 9.3. (a) Inhibition of subcutaneous U87MG human glioma cell xenograft (volume). Mice bearing U87MG tumours were treated with encapsulated BHK-neo (–□–), BHK-endo cells (–○–), or control (–△–). * P <0.01. (b) 21 days after treatment, U87MG tumours were harvested and weighed. P <0.001. (Reproduced with permission from *Nature Biotechnology*.)

Future perspective

Recent clinical trials with antiangiogenic inhibitors have not shown the efficacy that had been hoped for. The authors hypothesize that for angiogenesis inhibitors to be

effective, they must be given continuously for a longer period of time. These data support the need for novel delivery systems for antiangiogenic drugs. Cell-based delivery systems have the potential to overcome some of these issues. The progress made in the field of biomaterials, together with the field of genetic engineering, can be combined towards the development of this technology and other new delivery systems for local and systemic antiangiogenic therapy. The road to clinical studies using cell bioencapsulation is still facing problems, which need to be solved. Selection of cell type, cell genetic engineering, cell support, or polymer matrix, and immune aspects need to be taken in account and studied carefully to bring these antiangiogenic cell bioreactors to human clinical trials.

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