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Improving cell therapy – experiments using transplanted telomerase-immortalized cells in immunodeficient mice

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Abstract

Cell therapy is the use of stem cells and other types of cells in various therapies for age-related diseases. Two issues that must be addressed before cell therapy could be used routinely in medicine are improved efficacy of the transplanted cells and demonstrated long-term safety. Desirable genetic modifications that could be made to cells to be used for cell therapy include immortalization with hTERT (human telomerase reverse transcriptase). We have used a model for cell therapy in which transplantation of adrenocortical cells restores glucocorticoid and mineralocorticoid hormone levels in adrenalectomized immunodeficient mice. In this model, clones of cells that had been immortalized with hTERT were shown to be able to replace the function of the animals' adrenal glands by forming vascularized tissue structures when cells were transplanted beneath the capsule of the kidney. hTERT-modified cells showed no tendency for neoplastic changes. Moreover, a series of experiments showed that hTERT does not cooperate with known oncoproteins in tumorigenesis either in adrenocortical cells or in human fibroblasts. Nevertheless, hTERT was required for tumorigenesis when cells were implanted subcutaneously rather than in the subrenal capsule space. Changes in gene expression make hTERT-modified cells more robust. Understanding these changes is important so as to be able to separately control immortalization and other desirable properties of cells that could be used in cell therapy. Alternatively, desirable properties of transplants might be provided by co-transplanted mesenchymal cells: mesenchymal cell-assisted cell therapy. For both hTERT modification and mesenchymal cell-assisted cell therapy, genomics approaches will be needed to define what genetic modifications are desirable and safe in cells used in cell therapy.

1. Introduction

Many scientists, medical experts, and lay people see a strong future for cell therapy, i.e. the use of stem cells and other types of cells in various therapies for age-related diseases (Joseph and Morrison, 2005; Cao et al., 2005; Murry et al., 2006). Two issues that must be addressed before cell therapy could become routinely used in medicine are improved efficacy of the transplanted cells and demonstrated long-term safety of these procedures. Over the past few years work in this lab has been concerned with both of these issues.

The issues of efficacy and safety need to be considered in the context of the ways in which cell therapy would actually be used in clinical practice. In some forms of cell therapy autologous

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cells could be used, but if stem cells (adult- or embryo-derived cells) are to be employed the ideal would be to have a single qualified line available for use when required. In this case cells could be used clinically like drugs – an "off-the-shelf" approach in which cells that are capable of exerting a therapeutic effect, having been qualified as effective and safe, are widely available for immediate use. This would allow cell therapy to be a more widely available option in emergency situations, such as myocardial infarction (Murry et al., 2006). It would also allow the use of cells that have been genetically modified to improve their efficacy. Improved efficacy could, of course, include a lowered potential for immune rejection.

However, such forms of cell therapy must be demonstrably safe. Cell lines would have to have been demonstrated to be free of pathogens and not to be liable to long-term changes, such as neoplastic transformation, following introduction into the patient's body. The potential for the development of cancer has limited the use of genetically modified autologous bone marrow cells as therapy for genetic diseases (O'Connor and Crystal, 2006). The retroviral vectors that have been used for *ex vivo* genetic modification of bone marrow cells have a potential for insertional mutagenesis. Recent evidence suggests that lentiviral vectors could be less likely to activate unwanted gene expression in target cells than conventional retroviral vectors (Montini et al., 2006).

In all forms of therapy, whether based on the use of drugs, viral vectors or cells, the impact of adverse effects on the future of the therapy is enormous. Small numbers of serious side effects can make a drug, even though highly effective, unusable in clinical practice, both because of well-founded scientific concerns about safety and also because of concerns about legal liability for harm to patients caused by side effects. The future of adenoviral vectors has already been called into question based on the potential for severe patient reactions (O'Connor and Crystal, 2006). The safety issue is likely to dominate the question of the future acceptability and widespread use of cell therapy.

2. hTERT-immortalized cells for cell therapy

Since the pioneering experiments of Leonard Hayflick in the 1960s it has been known that the limited replicative capacity of human cells in culture is very unlikely to be an experimental artifact, but is a reproducible biological phenomenon (Hayflick, 1980). However, not until the discovery that the limitation in replicative capacity directly correlates with telomere shortening was the notion that it might be a culture artifact was finally laid to rest (Harley et al., 1990; Allsopp et al., 1992). Telomeres shorten in most dividing human somatic cells because of the lack of telomerase activity that is required for telomere maintenance (Greider, 1990; Harley, 1991). Telomere shortening caused by lack of telomerase activity also occurs in many cell types in human tissues in an age-related fashion (Hornsby, 2001).

Proof that the limitation on indefinite cell division in most human cells results from lack of expression of hTERT (telomerase reverse transcriptase) was obtained by showing that forced expression of hTERT is sufficient to immortalize normal human fibroblasts (Bodnar et al., 1998). Immortalization was accompanied by increased or stabilized telomere length, but cells retain a normal karyotype (Jiang et al., 1999; Morales et al., 1999).

Beginning with the first reports of hTERT-immortalization, it was speculated that this technology could be used to expand populations of cells for subsequent therapeutic transplantation (Bodnar et al., 1998; Weinberg, 1998; Morales et al., 1999; de Lange and DePinho, 1999). This was thought of as particularly important for the replacement of tissues and organs damaged during aging (Funk et al., 2000; Shay and Wright, 2000). In one proposed form of this therapy, cells with shortened telomeres would be isolated from a patient and telomere length restored by hTERT expression. The cell population would be expanded in culture and then cells would be reintroduced into the body to restore tissue and organ function.

In this scheme, hTERT plays a role in autotransplantation. hTERT-immortalization could also be useful in allotransplantation and xenotransplantation, by allowing expansion of cells with specific properties, such as stem cells or genetically modified cells.

As immortalization by hTERT became more widely studied, it became apparent that hTERT may exert effects on cells beyond extension of proliferative potential. In many cases hTERT-modified cells have widespread changes in gene expression, a topic discussed further below. Because hTERT both immortalizes and causes changes in gene expression that make cells more robust, as described in more detail below, hTERT-immortalized cells are particularly attractive for use in cell therapy and related technologies such as tissue engineering (Shay and Wright, 2000). A variety of hTERT-modified cells have been used in experimental cell therapy (Ulaner, 2004). A notable recent example is the construction of blood vessels engineered with hTERT-expressing smooth muscle cells (Klinger et al., 2006). Distinguishing the mechanisms by which hTERT affects cell proliferation versus cell function will be important so that the desirable features of each may be separately controlled.

3. Safety of hTERT-immortalized cells: Transplantation of normal and genetically modified adrenocortical cells

The model we have used for cell therapy is restoration of glucocorticoid and mineralocorticoid hormone levels in adrenalectomized immunodeficient mice by transplantation of adrenocortical cells (Thomas et al., 1997; Popnikolov and Hornsby, 1999; Thomas et al., 2000). Adrenocortical cells are co-transplanted with non-dividing 3T3 cells that play a supportive role in transplant formation, a topic discussed in more detail below. Here we review the progress made in this model and the problems that remain.

We have generated hTERT-modified bovine and human adrenocortical cells (Thomas et al., 2000; Suwa et al., 2001; Yang et al., 2001; Hornsby, 2004). For both bovine and human adrenocortical cells, we find that when culture conditions are optimized, ectopic expression of hTERT appears to be the only requirement for immortalization (Thomas et al., 2000; Suwa et al., 2001; Yang et al., 2001; Hornsby, 2004).

We transplanted bovine and human adrenocortical cells under the kidney capsule of immunodeficient mice. The subrenal capsule site is optimal for cell transplantation (Thomas et al., 1997), although other sites in the body can be used with more difficulty and less success (Popnikolov and Hornsby, 1999; Hornsby, 2004). The transplanted cells form a vascularized tissue structure that can replace the essential functions of the animals' own adrenal glands (Thomas et al., 1997; Thomas et al., 2000).

In our first set of studies on hTERT-immortalized cells we transplanted bovine adrenocortical cells that had been transfected with an hTERT plasmid (Thomas et al., 2000). The host mice were adrenalectomized. The transplant tissues that were formed beneath the kidney capsule resembled those formed from non-genetically modified cells with respect to size, histology, and rate of cell proliferation. The transplant tissue replaced the essential functions of the animals' adrenal glands by secreting cortisol and aldosterone (Thomas et al., 2000). In all animals the tissue showed no tendency for invasion into the kidney or other signs that could indicate malignant transformation.

At the time these hTERT-immortalized clones were created, we did not yet have access to an hTERT-expressing retrovirus. The hTERT-immortalized clones were created by cotransfection of cells with pCI-neo-hTERT, a plasmid in which hTERT is expressed from the CMV promoter, and pSV3neo, a plasmid containing the early region of SV40 virus that expresses both large T and small t antigens. We were forced to use cotransfection because we

found that stably transfected clones were not formed when the hTERT plasmid, or any other plasmids, were used without the cotransfection of pSV3neo. We theorized that this dependence on the inclusion of a plasmid encoding SV40 T/t antigens might result from direct adverse effects of the transfection process itself in primary cells (Hornsby, 2004).

Subsequently, we introduced hTERT alone into bovine and human adrenocortical cells using retroviral techniques (Thomas et al., 2002b). Despite the high level of telomerase activity in these cells and their probable immortalization, the cells did not form tissue as efficiently as cells that were hTERT-immortalized by cotransfection of pCI-neo-hTERT and pSV3neo. hTERT-expressing bovine cells were not able to form transplant tissues after being grown over long periods of time in culture, and hTERT-expressing human cells did not form tissue structures following cell transplantation (unpublished observations). These findings may indicate that SV40 T/t antigens expressed at low levels from the SV40 promoter may increase and/or stabilize differentiated functions in adrenocortical cells, in agreement with earlier work in cell culture (Cheng et al., 1989; Cheng et al., 1992). If this is so, the mechanism of action of SV40 T/t antigens should be clarified. For example, SV40 T antigen blocks the action of pRb, which by its involvement in heterochromatin formation could impair functional properties of stem cells and other cell types (Park et al., 2004).

In the experiments on introduction of hTERT by infection with retroviruses we also tested the potential cooperation of hTERT with known oncoproteins, SV40 T antigen and oncogenic Ras. The potential cooperation of hTERT with such oncoproteins in experimental tumorigenesis had been suggested by work in which SV40 T/t antigens, together with Ras, were unable to convert normal human fibroblasts into tumorigenic cells without the additional expression of hTERT (Hahn et al., 1999). However, in our experiments we found that SV40 T antigen and Ras without hTERT were capable of the conversion of both bovine and human adrenocortical cells into fully malignant cells without hTERT (Sun et al., 2004). Cells were invasive and metastatic. Subsequently we showed that human fibroblasts could also be converted to a fully malignant state by SV40 T antigen and Ras, without hTERT (Sun et al., 2005a) (Figure 1). In all cases, however, the tumors formed from the transplanted cells were not immortal and were telomerase negative. Eventually the tumors stopped growing and entered a phase of crisis and subsequent necrosis. Nevertheless, many of these tumors were capable of killing their hosts by the invasive and metastatic characteristics of the cells, before the cells reached crisis. If they were infected with an hTERT retrovirus before they reached crisis they became an immortal tumor line and their malignant properties were preserved indefinitely (Sun et al., 2004; Sun et al., 2005a).

One point should be noted here. Before performing the set of experiments described above, we had reported that bovine adrenocortical cells could be converted to a fully malignant state by retroviruses encoding SV40 T/t antigens, Ras, and hTERT, but not by SV40 T/t antigens together with Ras without hTERT (Thomas et al., 2002b). How could we have obtained this result when subsequently we showed unequivocally that hTERT was not required for neoplastic transformation? The explanation is almost certainly that whereas in later experiments we used retroviral co-infection without drug selection (Sun et al., 2004; Sun et al., 2005a), in the first set of experiments we performed serial retroviral infection/drug selection (Thomas et al., 2002b). Consequently, by the time SV40 T/t antigen/Ras-expressing cells had been derived as a pure population, they had undergone a substantial number of population doublings (PD). In recent studies we have shown that remaining PD alters the malignant properties of SV40 T antigen/Ras-expressing human fibroblasts. When SV40 T antigen and Ras were introduced into human fibroblasts with few remaining PDs, the cell population was unable to form tumors after transplantation (F. Yuan and P.J. Hornsby, unpublished observations). Although SV40 T/t antigen/Ras-expressing bovine adrenocortical cells had some remaining division potential

in these earlier experiments, the loss of division potential likely accounts for the failure of the cells to form tumors.

In summary, these studies on experimental cell transplantation of genetically modified cells show that hTERT does not cooperate with known oncoproteins in tumorigenesis. Only when telomeres have shortened to a critical level is telomerase activity needed for continued growth of the tumor. The question that should be considered is whether the latter conclusion would mean that hTERT modification could render cells more dangerous *in vivo* if perhaps subsequently they were to undergo mutational changes that caused activation of oncogenes. In all of the experiments we have performed using bovine and human adrenocortical cells, as well as human fibroblasts, we never observed any formation of tumors from cells modified by hTERT alone. Therefore, we can begin to form an estimate of the upper limit to the rate of neoplastic conversion of hTERT-modified cells. Further studies are needed to define this limit and thereby to determine more accurately the risks of using hTERT-modified cells in cell therapy. However, the fact that hTERT does not cooperate with known oncoproteins shows that hTERT is not an oncogene.

4. Approaches to improved cell transplantation using genomics

We also demonstrated that when oncogene-modified human cells are injected subcutaneously in immunodeficient mice they do, in fact, require hTERT to grow into a tumor, even though they do not when implanted in the subrenal capsule space (Sun et al., 2004; Sun et al., 2005a). Why was telomerase not required for tumorigenicity of cells implanted in the kidney? The subrenal capsule space has long been known as a "protective" environment in the body, suitable for the growth of embryonic rudiments and for tissue/cell transplantation (Povlsen et al., 1974; Giovanella et al., 1983). The critical features of the subrenal capsule microenvironment have not been definitively identified; important features may be the proximity to abundant capillaries within the kidney and a unique pattern of extracellular fluid flow from capillaries to lymphatics, giving the transplanted cells immediate access to adequate nutrients and oxygen before vascularization occurs. These features are probably lacking when cells are transplanted beneath the skin, except when care is taken to protect the cells from fluid loss (Popnikolov and Hornsby, 1999; Hornsby, 2004).

Presumably, therefore, hTERT confers additional properties on cells that enable cells to survive and grow in the subcutaneous space, which is in essence a harsh site for even the most robust cells to survive in. Proof that the action of hTERT is unrelated to telomere maintenance was obtained by studying cells that lack telomerase activity but nevertheless maintain telomeres via a process termed "alternative lengthening of telomeres" (ALT). Three ALT⁺ human SV40-immortalized fibroblast cell lines required only oncogenic Ras to be converted to a fully tumorigenic state, when tested for tumorigenicity by implantation beneath the kidney capsule of immunodeficient mice. Introduction of hTERT conferred strong telomerase activity but did not appreciably change the malignant properties of the cells. However, when cells were tested by subcutaneous injection, oncogenic Ras-transduced ALT⁺ cells did not form tumors, and in this site hTERT was required for tumorigenicity (Sun et al., 2005b).

hTERT-expressing cells show extensive changes in gene expression patterns (Smith et al., 2003; Cerezo et al., 2003). The ability of hTERT to exert a variety of effects that counteract cell death is striking (Sung et al., 2005) and these effects have been reported also in whole animals overexpressing TERT; in mice this protects against experimental heart failure (Oh et al., 2001) and has many other effects in the cardiovascular system (Serrano and Andres, 2004). hTERT-expressing cells are more resistant to chromosome damage caused by ionizing radiation (Pirzio et al., 2004; but see also Rubio et al., 2002; Rubio et al., 2004). hTERT has also been reported to be involved in the response of normal ("telomerase negative") cells to

double strand breaks at sites remote from telomeres (Masutomi et al., 2005). However, some anti-apoptotic effects of telomerase, e.g. prevention of apoptosis caused by activation of TRAIL (Dudognon et al., 2004) are hard to explain on the basis of any DNA repair mechanism. More confusingly, it has even been reported that hTERT inhibits p53-mediated apoptosis even in the absence of conferring telomerase activity (Rahman et al., 2005).

In conjunction with Dean Betts, we, in common with others, have used a genomics approach to determining what properties are conferred on cells by ectopic expression of hTERT (Perrault et al., 2005). The wide range of gene expression changes does not give unequivocal pointers as to the important changes conferred by ectopic hTERT that lead to improved cell survival and growth. Further studies will be valuable in determining these changes and using the information to improve transplantation of normal cells in cell therapy. It would be desirable to use hTERT to provide only immortalization and to confer other desired properties by separate specific genetic modifications.

5. Mesenchymal cell-assisted cell therapy

In most experiments on transplantation of normal and hTERT-modified adrenocortical cells, the cells were co-transplanted with 3T3 cells rendered incapable of further cell division. Our rationale was that the co-transplanted cells would provide support for angiogenesis in the transplant (Thomas et al., 1997; Thomas et al., 2002a). The particular line of 3T3 cells that we have used make a highly angiogenic secreted form of FGF-1 (Forough et al., 1993). The cells were treated with mitomycin C or lethal irradiation prior to cell transplantation.

The co-transplanted cells are not absolutely required. When adrenocortical cells are transplanted without 3T3 cells the tissue that forms at 36 days shows areas of necrosis, even though the rest of the tissue is apparently healthy and functional (Thomas and Hornsby, 1999). The necrotic area eventually resolves with the formation of healthy tissue about 70 days after introduction of the cells into the animal. When FGF-secreting 3T3 cells are included, the transplant tissue does not show a phase where necrosis is present.

Although the FGF secreted by the cells may be helpful, we later found that non-FGF-secreting 3T3 cells of various types also permit more rapid tissue formation when co-transplanted with bovine adrenocortical cells (unpublished observations). Indirect evidence suggests that the fact that the cells have been treated to render them incapable of division may be key to their effect (such treatment is necessary; if they are not so treated they overgrow the adrenocortical cells in the transplant). In retrospect, the supportive role of the 3T3 cells may be similar to the well-recognized role of fibroblasts, and mesenchymal cells generally, in assisting the growth of tumors from transplanted neoplastic cells (Bhowmick et al., 2004). Moreover, the state of the cells following treatment with mitomycin C or irradiation is in essence a form of premature senescence, a state that we now understand to be accompanied by the acquisition of a range of changes in gene expression that assist the growth of transplanted neoplastic cells (Campisi, 2005; D. Liu and P.J. Hornsby, unpublished observations).

Mesenchymal cell-assisted cell therapy offers another way to increase the efficacy of cell transplants. A genomics approach to understanding the apparently beneficial effects of placing the co-transplanted mesenchymal cells into a senescent state will allow the identification of specific genes that confer beneficial properties on the transplant. We now need to integrate findings on beneficial patterns of gene expression in co-transplanted mesenchymal cells with findings on beneficial patterns of gene expression conferred by ectopic hTERT and to use this information in future enhancements of cell therapy.

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Figure 1.

Invasive and metastatic behavior of primary (non-immortalized) human fibroblasts expressing SV40 large T antigen and oncogenic Ras without hTERT. (A) Two examples of tumors growing in the kidneys of immunodeficient mice, in one case (right) showing additional separate tumor masses within the peritoneal cavity. Photographs were obtained using blue light illumination for detection of green fluorescent protein (included in the retrovirus encoding Ras) together with low-level white light for visualization of the host organs. (B) Lung metastases visualized by fluorescence. (C) Hematoxylin and eosin–stained sections of two tumors showing invasion into the kidney; on the left is a section of a large tumor formed from CRL-2703 cells and on the right is a section of a small tumor formed from LF1 cells. (D) Tumor cells in the lung (above) and spleen (below) revealed by immunocytochemistry using an antibody against SV40 T antigen. Reproduced with permission from Sun et al., 2005a.