Cell Ablation in Transgenic Mice

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Introduction

Transgenic techniques make it possible to manipulate the mouse genome and to create new phenotypes. One of the applications of this techniques is the insertion of genes that lead to cell death by physiologically controlled expression of a toxin. The use of toxins in the study of normal biology is not a new idea, but the modern biotechnology enables the researcher to direct the toxin against specific cells or organs and the technique has advanced towards genetic surgery (O'Kane & Moffat 1992). Toxic substances coupled to monoclonal antibodies have been used in ablation of specific cells in the immunotoxin therapy of malignant diseases (Israel 1993, Neville et al. 1992). The genetically directed production of the toxins by toxigenes , however, makes it possible to produce cell death in specific tissues and cells even during embryonic development (Evans 1991).

Toxigenes are artificial genes that produce a gene product that is itself a toxin (»suicide genes«) or that result in a capacity to metabolize a non-toxic substance to a toxin, leading to the death of the host cell. There are several toxigene candidates for cell ablation: Diphtheria toxin A-subunit (DT-A), ricin A-subunit (R-A) and Herpes simplex virus type 1 thymidine kinase (HSV-tk) are the toxigenes used in cell ablation in living animals. Cell ablation studies have been extensively done not only in mice but with other species e.g. Drosophila has also been used (Kunes & Steller 1991, Moffat et al. 1992, Kalb et al. 1993). The natural toxin gene may be converted into a toxigene suitable for cell ablation by removing the sequence encoding the signal peptide (e.g. DT B-chain) to eliminate secretion allowing only cytoplasmic expression of the toxin. When DT-A or R-A is transcribed, the toxin kills the expressing cell immediately, but the HSV-tk needs to be externally induced by herpes drug treatment before the cell is affected. It is generally assumed that the toxic effect is limited to the expressing cells, allowing the elimination of these cells on the basis of their developmental stage and capacity for gene expression rather than their anatomical location. A general strategy for cell ablation in transgenic mice is to transfer a toxigene fused to a preferably cell-specific promoter and enhancer sequences into the genome to await gene activation.

Recently toxigene approaches have been applied in gene therapy of different tumors. It is obvious that cell ablation best suits therapeutic applications in somatic transgenic cells and cell lineage studies in transgenic animals, while the study of specific gene products in transgenic mice will profit most from the embryonic stem (ES) cell mediated gene knock-out approaches.

DT-A and R-A, suicide genes

The toxin producing gene tox of Corynebacterium diphtheria that causes human diphtheria is composed of two subunits. Without the B-subunit, that is recognized by a cell surface receptor, the A-subunit remains in the target cell. The A-subunit catalyzes in the cytoplasm the transfer of ADP-ribose from NAD to a modified histidine of eucaryotic elongation factor 2, resulting in the inhibition of protein synthesis and immediate cell death (Uchida 1982). In addition to inhibiting protein synthesis, DT-A may function as a nuclease (Chang et al. 1989). The structure of ricin (R) is analogous to that of DT. R-A acts as a highly specific ribonuclease that catalyzes the inactivation of the ribosomes (Lamb et al. 1985).

The suicide fusion gene strategy has been ap-

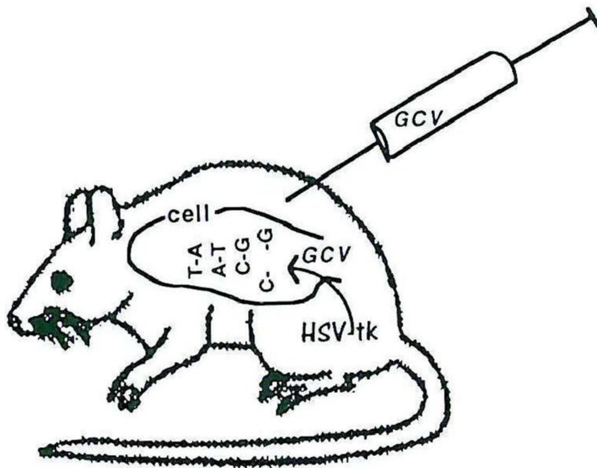

Figure 1. Induced ablation of cells. Herpes drug (Gancyclovir, GCV), administered to a transgenic mouse carrying HSV-tk transgene, is phosphorylated by HSV-tk enzyme to nuceloside monophosphates. These can be further phosphorylated by host enzymes, leading to inhibition of DNAsynthesis and cell death.

plied to cell ablation in several different types of tissues. Especially promoters that are expressed during adult life exclusively in cells, that are not vitally important for survival, and not expressed during the first days of early embryonic development, are suitable for studies on cell ablation. The elastase promoter, used in the early experiments, is transcribed almost exclusively in the exocrine cells of the pancreas (Palmiter et al., 1987). Expression of the toxigene controlled by the elastase regulatory signals might be expected to eliminate the developing exocrine cells in the transgenic mice at the stage where induction of elastase synthesis first occurs. The expression of the DT-A gene was associated with arrested pancreatic development and a 10- to 100-fold reduction in the number of islets and duct cells, with the complete absence of exocrine cells. The inheritance of this phenotype could not be studied due to the lethality of the phenotype in the founder mice. Morphological abnormalities, neonatal mortality and reproductive abnormalities have also been found in mice transgenic for DT-A driven by adipocyte lipid binding protein promoter (Homanics 1991). The lens crystallin regulatory elements combined to control toxigene expression is an excellent example of application of this technique. Crystallins are produced by lens fiber cells in a precisely coordinated spatial and temporal pattern during lens development. In the case of the $\gamma2$- and αA-crystallin genes, short regions upstream of the promoters are sufficient for the apparently physiologically regulated expression of attached promoters when introduced into the germline of transgenic mice. Breitman et al. (1987) used the DT-A coding sequence fused to the γ-crystallin promoter, and Landel et al. (1988) the RA-subunit coding sequence fused to the αA crystallin promoter to ablate lens structures in transgenic mice. In both cases, abnormalities were found only in the formation of the ocular lens, and thus lines of transgenic mice expressing the toxic phenotype could be established for further studies. Later studies showed that in transgenic mice which carried the DT-A coding sequence under the control of the α-crystallin promoter, the lens structures were transiently found during fetal life and disappeared in early postnatal life (Key et al. 1992).

The toxigene approach seems to be ideal in clarifying aspects of terminal differentiation and cell lineage, e.g. in the anterior pituitary among the various hormone producing cells. On the basis of the observation that some cell lines established from pituitary tumors produce both growth hormone and prolactin, and some cells producing both hormones can be detected in adult pituitary tissue, it was proposed that lactotropes and somatotropes may be derived from a common precursor. Fusion of the DT-A coding sequence with the rat growth hormone promoter resulted in transgenic founder mice lacking detectable circulating levels of growth hormone, impaired growth and almost complete absence of somatotropes in the pituitary. A severe decrease in the number of lactotropes accompanied the absence of somatotropes, suggesting a developmental relationship between

the two cell types (Behringer et al. 1988). Pituitary gonadotropes have been selectively and effectively eliminated in transgenic mice expressing the DT-A structural gene driven by bovine the α-subunit promoter sequence (Kendall et al. 1991). The phenotype of these transgenic mice was almost identical to the homozygous hpg spontaneous mutation. Interestingly, in these mice, all other pituitary functions were normal, with only a slight reduction in the number of lactotropes.

The gene product of DT-A is very toxic, even a single molecule may be sufficient to induce cell death (Yamaizumi et al. 1978). Attenuated forms of DT with reduced toxicity are also available, and have been successfully used in ablating cells of the ocular lens (Breitman et al. 1990). However, the toxicity resulting from transient expression of the transgene during early embryonic development might be expected to kill the embryo. These toxigenes are not ideally suitable for studying reproduction since no transgenic mouse lines can be formed unless an efficient and accurate method for controlled in vivo transgene modulation by site-directed recombination is used (Lakso et al. 1992). The failure to kill 100% of the targeted cells may represent the natural frequency with which cells fail to express a particular gene or may be due to modification events, methylation, deletion, or position effect. Also many genes expressed in early vertebrate embryos are duplicated, which is perhaps an evolutionary provision to ensure successful development in complex organisms. In gene knock-out studies it has been obvious that when one gene fails to function, by accident or design, another family member can take over (Lumsden 1990).

Thymidine kinase of Herpes simplex virus type 1, induced ablation of cells

An attractive alternative to the use of immediately toxic suicide genes involves the expression in the transgenic mice of genes that are not toxic as such but that may be induced by drugs to form toxic metabolites. HSV-tk is widely used as a selection marker in cell culture studies (e.g. ES cells) (Mansour et al. 1988). Thymidine kinase of Herpes simplex virus type 1 (HSV-tk) is not as such harmful to cells and therefore easily assayed from tissue homogenates and localized within tissues by in situ hybridization and immunohistochemistry. Nucleoside analogs, originally developed as herpes drugs, are metabolized by viral tk enzyme to cell toxic compounds, but are not recognized by the metabolic machinery of the mammalian cells. Gancyclovir, acyclovir and related drugs display little toxicity for normal mammalian cells at low dosage but block the replication of herpes virus (Tucker 1982, Miranda et al. 1982). Unlike mammalian thymidine kinases, HSV-tk is capable of phosphorylating these nucleoside analogs to nucleoside monophosphates, which can be further phosphorylated by host enzymes, leading to inhibition of DNA synthesis (Furman et al. 1980). Borrelli et al. (1988) first applied this model in transgenic mice expressing the HSV-tk protein from the immunoglobulin light-chain promoter and heavy chain enhancer elements; regulatory elements normally active in mature lymphocytes and their immediate progenitors. These transgenic mice expressed high levels of HSV-tk enzyme in the spleen and thymus and very low levels in other tissues. Offspring of transgenic founders were physiologically normal, but demonstrated a dramatic reduction in weight and atrophy of the spleen and thymus when treated with antiherpes drug. The drug treatment resulted in an estimated loss of 83% of cells in the thymus and 57% of cells in the spleen within one week. In another study antiherpes drug treatment of Ig-HSV-tk-carrying transgenic mice resulted in the elimination of practically all thymocytes (Heyman et al. 1989). If HSV-tk affects only DNA replication as originally hypothesized, the HSV-tk-mediated cell ablation technique depends entirely on cell division in the target tissue. Thus the immune system, where cells are dividing rapidly and continuously throughout the adult life of the mouse, is the

best target for HSV-tk-mediated cell ablation. However, (Wallace et al. 1991) produced transgenic mice with the bovine thyroglobulin promoter HSV-tk fusion gene, and by gancyclovir treatment of adult transgenic mice, they were able to ablate thyroid follicle cells, although these cells are nondividing in adult mice. This suggests that gancyclovir, an analog of guanosine, has several mechanisms of action as different guanosine phosphate derivatives play several key roles in cellular metabolism. On the other hand, gancyclovir may increase the rate of mitoses, but not sufficiently to account for the approximately 50% death of follicle cells. The mechanism of the »bystander effect« shown in gene therapy trials (Freeman et al. 1993) could explain some of the ablation results in transgenic mice as well. In tumors, containing a mixed population of HSV-tk somatic transgenic and nontransgenic cells and exposed to gancyclovir, the HSV-tk positive cells were lethal to HSV-tk negative cells both *in vitro* and *in vivo* .

In HSV-tk transgenic mice the most disappointing side effect has been the undesired testicular expression of the HSV-tk. This limits the use of the tk transgenic mice since in many studies transgenic males, especially those with a high copy number, are sterile (Al-Shawi et al. 1988, Al-Shawi et al. 1991, Braun et al. 1990, Heyman et al. 1989, Iwakura et al. 1988). It has been demonstrated that the HSV-tk itself, also devoid of any upstream regulatory sequences, is expressed in the testis, obviously directed by a another promoter located in the coding region of the gene (Al-Shawi et al. 1991). Without a testis specific promoter enhancer region, the HSV-tk was expressed in the developing testis and localized in the spermatogonium on day 7 after birth. However, in four independent lines of transgenic mice carrying bovine follicle stimulating hormone β-subunit (FSHβ) fused to HSV-tk, the males expressing HSV-tk in the testis, were fully fertile (Markkula et al. 1993). Contrary to other studies on HSV-tk fusion genes, in this study the promoter consisted of a 2.3 kb fragment of 5' sequence of bovine FSH β-subunit, the murine equivalent of which is endogenously expressed in the testis, and also the onset of the murine FSHβ and HSV-tk gene expression is similar (Markkula et al. unpublished).

The FSHβ-HSV-tk transgenic mice were originally designed for studies on the role of FSH in the pituitary-gonadal axis (Markkula et al. 1995). The HSV-tk transgene protein was colocalized in the same pituitary cells as the FSH β-subunit protein as determined by immunohistochemistry. The most pronounced ablation of gonadotropes was induced in utero when the drinking water of pregnant females was replaced with acyclovir solution on day 13 of the pregnancy. The transgenic newborn pups had significantly lower concentrations of pituitary gonadotropes (55-90%), and the plasma gonadotropin levels were reduced (30%). It was expected that both gonadotropins, FSH and luteinizing hormone (LH), would be affected by ablation induction with acyclovir treatment, since some pituitary gonadotropes are known to produce both FSH and LH. In the pituitary the expression of the endogenous FSHβ (Dihl et al. 1988) and obviously also that of the transgenic FSHβ-HSV-tk, is initiated during fetal life, several days earlier than the postnatal testicular expression of those genes. Because of this, the mouse model may be used in gonadotrope cell differentiation and maturation studies. Ablation induction in castrated adult males also significantly reduced the levels of circulating FSH after one and two weeks of gancyclovir treatment, but the possible gonadotropin ablation caused by the treatment (Markkula et al. 1995) was masked by the general postcastration reduction in the pituitary gonadotropin concentrations (Rodin et al. 1990).

Modification of endogenous genes in transgenic mice

Gene targeting in ES cells provides a powerful tool for generating mice carrying designed mutations in the germ line (Thomas & Ca-

pecchi 1987, Bradley et al. 1992). Approaches to gene inactivation commonly involve the introduction of a null mutation directly into ES cells from which a homozygous mutant mice can be generated. Because the null mutation is carried in the germ line of the mutant mice, it will exert its effects from the onset of the promoter activity. Although this approach to gene inactivation is valuable for many applications it is important that the inactivation of the particular gene occurs in a conditional manner – for instance, in a predefined cell lineage or at a certain stage of development. Such conditional gene targeting would not only overcome problems posed by the fact that null mutations in the germ line are often lethal, but would also allow a more precise analysis of the impact of a mutation on individual cell lineage.

In a recent work Gu et al. (1994) used an enzyme called Cre recombinase from bacteriophage P1, a virus that infects *Eschericia coli.* In infection Cre separates any phage genomes that become joined to one other. To do this Cre lines up short sequences of phage DNA called loxP sites and removes the DNA between them, leaving one lox site behind. The system was first studied in cells *in vitro* but soon it was shown that Cre is effective in mammals *in vivo*, and could be used with high efficiency to excise chromosomal DNA (Orban et al. 1992) or to turn on a foreign gene in transgenic mice (Lakso et al. 1992). In principle, Cre-loxP-mediated gene targeting should allow the inactivation of any gene in any tissue at any stage of development, and also cause specific cell ablation.

Ablation of somatic transgenic cells

Today the ability to effectively transduce large numbers and diverse populations of somatic cells provides scientists with a rapid and reproducible source of animals for the analysis of the function of genes transferred and expressed *in vivo*. Somatic transgenic mice could also create new opportunities for direct studies of the effects of transgene expression at different developmental stages, or transgenes whose expression is lethal during embryogenesis, or even multiple transgenes could be expressed in series in the same animal (Zhu et al. 1993).

Somatic gene transfer paves the way for gene therapy, selective ablation of e.g. tumor cells. The combination of toxigenes, the Cre-loxP - system and direct gene transfer into tissues *in vivo* has many potential tantalizing applications. The toxigenes offer a highly specific therapeutic agent, provided a sufficiently stringent regulation of its expression can be imposed. Tumors that are made up of rapidly diving cells invading the nonproliferating tissue are good targets for HSV-tk-mediated gene therapy. Barba et al. (1993) have been able to establish brain tumors by intra-cerebral injection of HSV-tk-modified tumor cells (Fisher 344 rat brain tumor model) in which tumor cells were ablated by gancyclovir treatment. In survival studies, all rats with tumors treated with gancyclovir survived for 90 days while all untreated rats died within 25 days. Surprisingly, it was found that tumors could be eliminated by gancyclovir treatment even when only 50% of tumor cells carried the HSV-tk. The use of HSV-tk gene therapy to carry out conditioned killing of cells was first suggested by Moolten & Wells (1990). The bystander effect in which the phosphorylated gancyclovir could be transferred from cell to cell, may provide an additional improvement to the effectiveness of HSV-tk - mediated gene therapy experiments that have now been applied widely to different types of tumors (Vile & Hart 1993, Freeman et al. 1993, Ram et al. 1993, Caruso et al. 1993).

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Summary

The toxigene approach, in which the toxic gene product is guided by the cell specific promoter to the desired tissue, is an effective way of ablating cells both in germline transgenic mice and also in somatic transgenic tissues. It opens up new possibilities for physiological and cell lineage studies and gene therapy of tumors. The more effective targeting of toxigenes into the genome and timing

of their expression will further increase their applicability.

Yhteenveto

Solujen tappaminen myrkkygeeni-menetelmällä, joissa tietylle solulle spesifisen geenin säätelyosa on yhdistetty myrkkyä muodostavan geenin rakenneosaan, on erittäin tehokasta sekä perimältään siirtogeenisissä hiirissä että siirtogeenisiksi tehdyissä kudoksissa. Se avaa aivan uusia mahdollisuuksia tutkia solujen fysiologiaa, solulinjoja sekä hoitaa tuumoreita geeniterapian avulla. Myrkkygeenien ohjaaminen tiettyyn kohtaan perimässä ja vaikutuksen tarkka ajoittaminen lisäävät menetelmän sovellutuksia.

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