Letter to the Editor

HIGH EFFICIENCY LIPOSOME-MEDIATED TRANSFECTION OF THE TYROSINASE GENE TO CULTURED CELLS: A MODEL FOR THE GENE THERAPY OF HAIR COLOR RESTORATION

Dear Editor:

Hair pigmentation is a function of the tyrosinase gene. Lloyd et al. (1987) found that the level of tyrosinase correlated with hair color, with the lowest activity occurring in blond hair bulbs and the highest in red hair bulbs. However, most important, Lloyd et al. (1987) found that the human tyrosinase antigen was absent in white hair bulbs. Thus the loss of tyrosinase seems to be the basis of hair turning white.

To further study problems of alapceia and hair color loss we have developed a system of collagen-sponge-gel-supported histoculture of hair-producing skin (Li et al., 1991, 1992a, 1992b, 1992d, 1993a). In the skin histoculture system, hair can grow at in vivo-like rates (Li et al., 1992a). We have also observed that liposomes can specifically target hair follicles in the skin histocultures to deliver products into the follicle (Li et al., 1992c, 1993a and 1993b). In the 1970s we constructed the first DNA-liposomes (Huffman et al., 1973). Such DNA-containing liposomes were recently found to also specifically target high molecular weight DNA into the hair follicle of histocultured skin (Li et al., 1993b). These data indicated gene transfer to the hair follicle was feasible and thus gene therapy of the hair process may be possible.

In this light we took advantage of the existence of the full-length human tyrosinase cDNA clone isolated by Shibahara et al. (1988) and Takeda et al. (1989). This group transfected the tyrosinase gene with the calcium phosphate method to the mouse amelanotic cell line K1735. Approximately 1.4% of the cells were successfully transfected as evidenced by a positive dopa oxidation reaction. In addition, tyrosinase hydroxylase activity was detectable in the transfected cells. We report here that liposome-mediated gene transfer greatly enhanced the transfection frequency of the human tyrosinase cDNA clone.

Positively charged liposomes were prepared by freezing and thawing. A total of 20 mg of lipid in a ratio of 5:3:2 of phosphatidylcholine (PC):cholesterol (Chol):phosphatidylethanolamine (PE) were rotary evaporated for 1 h with a vacuum drier from a chloroform solution to form a thin film on the walls of a 5-ml round-bottomed flask. The dried, thin film lipid was suspended in 2 ml phosphate buffered saline (PBS, pH 7.4) on a vortex mixer and then sonicated with a Branson probe-type sonicator fitted with a microtip at power level 3 for 8 min. Then 200 µg of the plasmid pH90HT2 containing a full length human tyrosinase cDNA (Shibahara et al., 1988; Takeda et al., 1989) were entrapped in the above suspension by water bath sonication for 2 min followed by freezing and thawing 3 times.

The human fibroblast cell line FS-3 and mouse amelanotic melanoma cell line K1735 were precultured in 60-mm culture dishes with Eagle's MEM + 10% fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium + 10% FBS, respectively, for 24 h. The fibroblasts and K1735 cells were transfected by incubating the cells with 0.5 ml of the liposome-tyrosinase cDNA solution and 1.5 ml Eagle's MEM or DME medium + 10% FBS in each dish for 48 h. Then the fibroblasts and K1735 cells were further incubated for another 7 and 3 days, respectively, after aspirating the liposome-tyrosinase cDNA solution. The fibroblast and K1735 cells were then harvested by trypsin digestion and centrifuged at 800 x g for 5 min to attach them to cytopsin slides. Fifty micrograms of naked-tyrosinase-cDNA solution with Eagle's MEM served as the control and were also incubated with the two cell types as described above.

The dopa-oxidase reaction and immunohistochemical staining for tyrosinase were used to detect tyrosinase expression in the transfected fibroblasts and K1735 cells. For dopa oxidase, the cytopsin slides of fibroblasts and K1735 cells, as described above, were incubated with 1 mg/ml of L-dopa solution in PBS (pH 7.4) as described by Kugelman and Van Scott (1961) for 12 h in 37°C. Then the cytopsin slides were counterstained by hematoxylin and eosin. Tyrosinase-(dopa oxidase)-positive cells were then microscopically identified.

The Dako LSAB (labeled streptavidin-histin) kit was used for immunohistochemical staining of tyrosinase-containing cells. The primary antibody was rat anti-human tyrosinase monoclonal antibody TMH1 (Tomita et al., 1985; Jiménez et al., 1988). First, the fibroblasts and K1735 cells in the cytopsin slides were fixed in

| TABLE 1 |
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| **COMPARISON OF TRANSFECTION EFFICIENCY OF HUMAN TYROSINASE BY CALCIUM PHOSPHATE AND VIA LIPOSOMES** |
| **Method** | **Percent of Transfected Cells** | **Method of Detection** |
| Calcium phosphate | 1.4 (K1735 cells) | Dopa-oxidase reaction and tyrosinase hydroxylase activity |
| Liposome mediated | 52.3 (FS-3 cells) | Dopa-oxidase reaction and tyrosinase immunohistochemical staining |
|  | 52.0 (K1735 cells) | |

* Data are from Takeda, A.; Tomita, Y. et al. (1989). For the liposome-mediated transfection, the percent of transfected cells is based on the counting of 1200 cells of each dopa-oxidase reaction and tyrosinase immunohistochemical staining slide under the microscope.
acetone for 10 min and air dried. Serial incubations were then performed with hydrogen peroxide, blocking serum, primary antibody (1:400), linking antibody, peroxidase-conjugated streptavidin, and 3-amino-9-ethylcarbazol substrate solution for 10 min each. The slides were then lightly counterstained with Mayer’s hematoxylin and mounted with liquid glycerol gelatin (DAKO). A frozen section of human melanoma tissue was used as a positive control. The negative control was carried out by replacing the primary antibody with PBS.

Figures 1 A,C,D and 2 A,C show tyrosinase expression in the liposome-mediated transfected fibroblasts and K1735 cells detected by the dopa oxidase reaction and by tyrosinase immunohistochemical staining, respectively. The tyrosinase-positive cells are clearly identified by black pigmented cytoplasmic granules (dopa oxidase positive) and red cytoplasmic granules (tyrosinase immunohistochemical staining). Note the extensive and high efficiency of liposome-mediated transfection of the human tyrosinase cDNA into the fibroblasts and mouse K1735 cells. The percent of cells transfected was approximately 52.3% of the fibroblasts and 52% of the K1735 cells. Figures 1 B,E and 2 B,D are the control treated with naked-tyrosinase cDNA. Note that both the dopa oxidase reaction and tyrosinase immunohistochemical staining are negative.

Table 1 demonstrates the efficiency of liposome-mediated transfection of human tyrosinase cDNA compared with the calcium phosphate method. The data for calcium phosphate method of tyrosinase cDNA transfection are taken from Takeda et al. (1989). As Table 1 shows, the efficiency of liposome-mediated transfection is 50 times more than the calcium phosphate method.

Recently, Zhu et al. (1993) found that intravenous injection of plasmid-liposome complexes can efficiently transfect all tissues of adult mice. Thus liposome-mediated transfection is a powerful tool. Future experiments in this laboratory will focus on attempts to transform white hair follicles in skin histoculture as well as in vivo with the liposomally-entrapped tyrosinase clone.
Fig. 2. Human fibroblasts (A) and mouse melanotic melanoma cell line K1735 (C) on a cytopsin slide after transfection by liposomally entrapped human tyrosinase cDNA for 48 h and further culturing for 7 and 3 days, respectively. Expression of human tyrosinase gene was detected by tyrosinase immunohistochemical staining and counterstaining with Mayer’s hemotoxylin. Tyrosinase positive cells are identified by red cytoplasmic granules. Note the high efficiency of transfection of human tyrosinase cDNA into the fibroblasts and K1735 cells. (B) and (D) are controls with the fibroblasts (B) and K1735 cells (D) treated with naked cDNA. Note no tyrosinase can be detected. Light microscopy. X500.

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