ESTABLISHMENT OF A TUMOR-ASSOCIATED MACROPHAGE CELL LINE (TtT/M-87) FROM MOUSE THYROTROPIC PITUITARY TUMOR

KINJI INOUE¹, AKIHIRO ITO², YAE KUBOTA³ and SATONORI KURASHIGE³

¹Department of Morphology, Institute of Endocrinology, Gunma University, Maebashi, Gunma 371,

²Department of Cancer Research, Research Institute for Nuclear Medicine and Biology, Hiroshima University, Hiroshima 734, and ³College of Medical Care and Technology, Gunma University, Maebashi, Gunma 371, Japan

ABSTRACT

Cells derived from a thyrotropin-producing tumor (TtT) arisen from a radio-thyroidectomized mouse were cultured for 2 years. After this long period of cultivation, we unexpectedly established a mononuclear macrophage cell line (TtT/M-87). Cell proliferation and survival of TtT/M-87 were completely dependent on the conditioned medium of fibroblast or the L929 cell line, which is known to secrete a colony-stimulating factor. TtT/M-87 is polymorphic and has a ruffled membrane. TtT/M-87 shows strong phagocytotic activity. The karyotype is diploid ($2n=40$) similar to that of the normal mouse cells. Electron microscopy shows that TtT/M-87 contains many lysosomes in its cytoplasm and the cell surface is surrounded with pseudopodia. No viral particle was observed. The appearance of TtT/M-87 resembles the general characteristics of macrophage, and TtT/M-87 is therefore considered to be a macrophage cell line derived from tumor-associated macrophage.

The progress of immunology has let the function of macrophages being understood more clearly, with many important functions related to the immune system being suggested. The relationship between macrophages and tumors has also been discussed (7, 9). Some macrophage cell lines have already been established and used for the study of macrophages (10). However, almost all of these cell lines have been established from a naturally transformed macrophage tumor (11), or artificially transformed by infection with the transforming virus SV 40 (8, 12). Therefore, these cell lines have partially lost the normal characteristics of the macrophage, such as dependence on colony-stimulating factor (CSF) or their karyotype. For a long period we cultivated cells derived from a transplantable thyrotropic

tumor, which produces thyrotropin. During this cultivation we unexpectedly established a cell line with characteristics of the macrophage. In this paper we describe this newly established macrophage cell line, together with its morphological characteristics and mode of proliferation.

MATERIALS AND METHODS

Pituitary Tumor and Cell Culture

The transplantable thyrotropic tumor used for cultivation has previously been established from a pituitary tumor, which had arisen from radio-thyroidectomized F₁ mice (C57BL/6N X C3H/2N) in 1984 (5). On 19 September 1985 the thyrotropic tumor was obtained from a tumor-bearing mouse. The thyrotropic tu-

mor was rinsed with Hank's balanced salt solution devoid of Ca and Mg ions and minced into small pieces. The minced tissue pieces were then incubated with 1% collagenase for 30 min at 37°C. Following the incubation with collagenase the tissue pieces were dispersed mechanically by trituration with a pipette. The dispersed cells were seeded in plastic culture flasks and incubated in 95% air and 5% CO₂ at 37°C. The medium was exchanged every 3 days. Ham F12, supplemented with 15% fetal calf serum, 0.01% kanamycin and 0.035% sodium bicarbonate was used for primary cell culture.

L929 Cell Conditioned Medium

L929 cells were cultured with Ham F12 supplemented with 5% fetal calf serum. Conditioned medium of L cells was harvested every 5 days and kept at -20°C until used for maintaining macrophage cell line. The macrophage-stimulating factor in the conditioned medium of L cells was condensed partially by salting out with ammonium sulfate. Briefly, 50 g ammonium sulfate was dissolved into 100 ml conditioned medium of L929 at pH 4.5. The formed precipitate was dissolved in distilled water and dialysed in phosphate-buffered saline.

Colony Cloning

The macrophage colonies were separated from other neighboring cells by scraping them from the culture dishes with a platinum needle and then sucking them with a pipette. They were subcultured until they grew and formed new colonies. Colony clonings were repeated until all the cells showed a similar morphological appearance without any other contaminating cells. After obtaining sufficient colonies from the one cell type, they dissociated with 0.5% trypsin for 20 min. The dissociated cells were seeded with Ham F12 or alpha modified MEM, which were supplemented with 15% fetal calf serum and 20% conditioned medium of L929 cells under 5% CO₂ and 95% air at 37°C.

Electron Microscopy

The cells cultured on a plastic dish were fixed

with 2.5% glutaraldehyde buffered with 1/15 M phosphate buffer at 4°C for 1 h. Following the glutaraldehyde fixation the cells were harvested with a rubber policeman and then post-fixed with 1% OsO₄ and 4% sucrose containing 1/15 M phosphate buffer (pH 7.4) at 4°C for 1 h. After dehydration with an ascending ethanol series they were embedded in Epon-Araldite and ultrathin sections were made. In order to observe phagocytosis, polyhedra prepared from silkworm infected with cytoplasmic polyhedrosis virus were used as a marker for phagocytosis. The polyhedron of the insect polyhedrosis virus is a hexagonal crystalline protein (about 2 µm in diameter) composed of the virus particles (6). The typical appearance of the polyhedron is a good marker for electron microscopic observation of phagocytosis and the processes of intracellular lysis of phagocytosed protein (4).

Karyotype

The karyotype was analyzed by a conventional method (3). Briefly, cells in exponential growth phase were treated with colchicine for 2 h and were then dispersed with trypsin and treated with sodium citrate to stimulate cell lysis. Following fixation with a 3:1 mixture of methanol and glacial acetic acid, cells were placed on glass slides, dried quickly and the chromosomes were stained with Giemsa's solution (Merck, F.R.G.)

RESULTS

Establishment of Macrophage Cell Line

At the start of cultivation, at least three kinds of cells were identified by phase contrast microscopy: thyrotropic tumor cells characteristically showing an epithelial appearance; fibroblasts; dendritic cells with irregular cell processes. The epithelial cells were very difficult to culture and disappeared after the several subcultures. The third cell type proliferated well but their proliferation was associated intimately with fibroblast growth. Ten months after the start of cultivation, the third cell type showed very rapid growth, although cell proliferation was still dependent on growth of the fibroblasts. Therefore, we used medium containing 20% L929 conditioned medium which is known to contain a macro-

Fig. 1, A and B Phase-contrast micrographs of established macrophage cell line (TtT/M-87), showing dendritic cell-like appearance with many cell processes. Some refractile light bodies are seen in the cytoplasm. Micrographs show the TtT/M-87 at low (A) and high (B) cell population densities.

phage-colony stimulating factor (13). Use of this medium led to a satisfactory proliferation of the third cell type and the foundation of colonies composed of cells of a single type. These colonies were separated carefully from contaminating fibroblasts with a platinum needle and subcultured. By repeated colony cloning, the third cell type was purified into a single kind of cell. After 80 subcultures over a period in excess of one year, the growth rate and morphological characteristics of this cell type were unchanged. It was therefore considered that a cell line named TtT/M-87 was established. TtT/M-87 cells showed a dendritic appearance having ruffled membranes with many long cell processes. Phase-contrast microscopy showed refractile vacuoles in the cytoplasm (Fig. 1). Observation with time-lapse video showed that cells of TtT/M-87 generally had a hard contact with the culture dish at each cell center and were not particularly motile. However, cell processes were seen to move actively by repeated elongation and shrinking in many directions. TtT/M-87 is a mononuclear diploid cell and the karyotype is $2n=40$ (Fig. 2). They have

Fig. 2 Example of chromosomes of TtT/M-87; $2n=40$ as in macrophage from normal mice

Fig. 3 Electron micrograph of a TtT/M-87 cell. Note many electron-dense lysosomes (L) in the cytoplasm and many pseudopodia on the cell surface (arrows). $\times 7,000$

Fig. 4 Electron micrograph of a TtT/M-87 cell during mitosis, showing chromosomes (C) and many lysosomes (L) in the cytoplasm. $\times 7,000$

Fig. 5 Electron micrograph showing phagocytotic activity of TtT/M-87 demonstrated with polyhedra of cytoplasmic polyhedrosis virus of silkworm. Note the phagocytosed polyhedra in the cytoplasm (CP) and a polyhedron trapped by pseudopodia (TP). A well-developed Golgi apparatus (G) with Golgi vesicles are seen in the cytoplasm. $\times 10,000$

Fig. 6 Mode of cell proliferation of TtT/M-87, showing satisfactory proliferation in the medium containing conditioned medium of L cells, but not in its absence. Maximum growth of TtT/M-87 was obtained in the medium containing 20% conditioned medium. Concentrations % of conditioned media of L cell are indicated.

strong phagocytotic activity and phagocytosed substances added to the medium, such as latex beads or polyhedra of the cytoplasmic polyhedrosis virus of silkworm. These characteristics of TtT/M-87 are similar to those of normal macrophages. TtT/M-87 cells were back-transplanted into syngeneic mice, but they did not show any tumorigenicity.

Electron Microscopy and Phagocytosis by TtT/M-87

Electron microscopic observation of TtT/M-87 showed many pseudopodia on the cell surface and many polymorphic electron-dense lysosomes in the cytoplasm (Fig. 3). Lysosomes were also observed in mitotic cells (Fig. 4). A well-developed Golgi apparatus with many Golgi-associated vesicles were seen (Fig. 5). No virus particles were seen in TtT/M-87.

For observation of phagocytosis by TtT/M-87, the polyhedra of cytoplasmic polyhedrosis virus of silkworm were used as a marker. Soon

after the addition of polyhedra, phagocytosis by TtT/M-87 occurred (Fig. 5).

Growth of TtT/M-87

Proliferation of TtT/M-87 is completely dependent on the presence of fibroblast-conditioned medium. As shown in Fig. 6, TtT/M-87 proliferated well in the medium which contained 20% L929 cell-conditioned medium in Ham F12 supplemented with 15% of fetal calf serum. The doubling time of TtT/M-87 was about 24 h in logarithmically growing cultures.

DISCUSSION

Some macrophage cell lines have already been established. Almost all of them were, however, established by artificial transformation using the transforming virus SV 40 (12) or a naturally transformed macrophage tumor (11). The characteristics of these cell lines are therefore different from those of naturally obtained macrophage, with respect to the dependence on a growth factor or the karyotypes. Our present cell line, TtT/M-87 shows macrophage-like characteristics such as dependence on a colony-stimulating factor for growth, strong phagocytic activity and morphological features such as many lysosomes in the cytoplasm and pseudopodia on the cell surface. Furthermore, it is recognized that TtT/M-87 cell line has a Mac-1 antigen that generally exists in murine macrophages which can stimulate *in vitro* lymphocyte response through the production of interleukin 1-like substance (unpublished, S. Kurashige *et al.*). Therefore, TtT/M-87 is considered to be a macrophage cell line. TtT/M-87 was derived from a pituitary tumor, and hence it may be originated from a tumor-associated macrophage. This newly established macrophage cell line TtT/M-87 shows a normal karyotype and cell proliferation is completely dependent on a colony-stimulating factor. These indicate that the general characteristics of macrophages are well preserved in TtT/M-87. Therefore, this cell line may facilitate research on macrophages. The reason for the appearance of considerably large number of macrophages in the thyrotropic tumor is still unknown. It has recently been shown that pro-

lactin stimulates tumoricidal macrophages (2) and that interleukin produced from a macrophage has broad stimulatory action on the pituitary function (1). These reports suggest that some relationship may exist between the pituitary gland and macrophages.

The authors wish to thank Professor K. Kurosumi, Gunma University, for his valuable suggestions.

Received for publication 8 July 1988

REFERENCES

1. BERNTON E. W., BEACH J. E., HOLADAY J. W., SMALLRIDGE R. C. and FEIN H. G. (1987) Release of multiple hormones by a direct action of interleukin-1 on pituitary cells. *Science* **238**, 519-521
2. BERNTON E. W., MELTZER M. S. and HOLADAY J. W. (1988) Suppression of macrophage activation and T-lymphocyte function in hypoprolactinemic mice. *Science* **239**, 401-404
3. HSU T. C. (1973) Karyology of cells in culture. In *Tissue Culture* (ed. KRUSE R. F. and PATTERSON M. K.) Academic Press, New York/London, pp. 764-777
4. INOUE K. (1974) Phagocytosis of nuclear polyhedra and the localization of the acid phosphatase in the granular cell of the silkworm, *Bombyx mori* L. *J. Sericult. Sci. Japan* **43**, 394-400 (in Japanese with English summary)
5. ITO A., NAITO M., KAWASHIMA K. and WATANABE H. (1984) Establishment of transplantable thyrotropic pituitary tumor in radiothyroidectomized BDF₁ mice. *Nagasaki Med. J.* **59**, 428-432 (in Japanese)
6. KOBAYASHI M. (1971) Replication cycle of cytoplasmic-polyhedrosis virus as observed with the electron microscope. In *The Cytoplasmic-Polyhedrosis Virus of the Silkworm* (ed. ARUGA H. and TANAKA Y.) University of Tokyo Press, Tokyo, pp. 103-128
7. MILAS L., WIKE J., HUNTER N., VOLPE J. and BASIC I. (1987) Macrophage content of murine sarcomas and carcinomas: Associations with tumor growth parameters and tumor radio-curability. *Cancer Res.* **47**, 1069-1075
8. MORGAN C., POLLARD J. W. and STANLEY E. R. (1987) Isolation and characterization of a cloned growth factor dependent macrophage cell line, BAC1.2F5. *J. Cell. Physiol.* **130**, 420-427
9. MUKAI M., SHINKAI K., TATEISHI R., MORI Y. and AKEDO H. (1987) Macrophage potentiation of invasive capacity of rat ascites hepatoma cells. *Cancer Res.* **47**, 2167-2171
10. RALPH P. (1981) Continuous macrophage cell lines—Their use in the study of induced

- and constitutive macrophage properties and cytotoxicity. In *Lymphokines* (ed. Pick E.) Vol. 4, Academic Press, New York, pp. 175-195
11. RALPH P. and NAKOINZ I. (1975) Phagocytosis and cytolysis by a macrophage tumour and its cloned cell line. *Nature* **257**, 393-394
 12. SCHWARZBAUM S., HALPERN R. and DIAMOND B. (1984) The generation of macrophage-like cell lines by transfection with SV40 origin defective DNA. *J. Immunol.* **132**, 1158-1162
 13. STANLEY E. R. (1985) The macrophage colony-stimulating factor, CSF-1. *Meth. Enzymol.* **116**, 564-587