which binds a number of lectins and is associated with the membrane matrix (in preparation). Crossed electrophoresis in the absence and presence of reducing agents reveals 2 major polypeptides with abnormal migration behavior and apparent molecular weights of 37 and 69 KD, both of which exhibit a considerably reduced electrophoretic mobility in the absence of 2-mercaptoethanol. Under reducing conditions these peptides migrate close to the monomers and dimers of opsin (mol.wt 35 KD and 70 KD), but are clearly distinct as becomes apparent after 2-dimensional electrophoresis (fig., c). Opsin is characterized by its tendency to form oligomers by hydrophobic interactions under any electrophoretic conditions[9] and by its property to form ill-defined banding patterns. This electrophoretic behavior was used to assess the coordinates of opsin and its oligomers in 2-dimensional gels and to estimate approximate molecular weights of other components in relation to opsin (fig., c). The figure c also demonstrates that opsin will undergo renewed polymerization and depolymerization upon re-electrophoresis in a second dimension. In non-reducing electrophoresis both 37 and 69 KD peptides show increased mobility (apparent mol.wts: 34 KD and 60 KD) and migrate well ahead of opsin and its dimer, thereby indicating an asymmetric molecular shape. The larger of these peptides is possibly identical with the 60 KD band seen in 2-dimensional electrophoresis without reducing agent (fig., b), whereas the smaller one is not well enough resolved from the monomeric opsin band in 1-dimensional techniques. Both peptides do not appear to be composed of smaller subunits but they may be related polypeptides and are probably intramolecularly bonded via disulfide bridges. Under nonreducing electrophoretic techniques a molecular asymmetry caused by a disulfide bridge would result in a higher electrophoretic mobility (right hand side of fig., c) which is sensitively recognized using gradient gels[6]. The 37 KD peptide is probably identical with a component of similar molecular weight which is resolved in 2-dimensional isoelectric focusing and exhibits an isoelectric point slightly more acidic than opsin[4] which, however, is irrelevant for electrophoretic behavior under denaturing conditions. Both the quantitative loss of polypeptides on polyacrylamide gels without prior reduction and the existence of 2 peptides with apparent intrasubunit disulfide linkages in the photoreceptor membrane sufficiently explain the ultrastructural observation of rapid disorganization after treatment with sulfhydryl-reducing agents. The selective loss of a complex 132 KD glycoprotein from the detergent-lysed mixture of photoreceptor polypeptides in the absence of reducing agents during electrophoresis also indicates that this protein may participate in a disulfide-based supramolecular structure. It has been reported however, that such high molecular weight complexes can form randomly between sulfhydryl groups of unrelated proteins if they are solublized in the absence of an antioxidant but it was also shown that this can be prevented effectively by either blocking native sulfhydryl-groups chemically or by carefully excluding oxygen from all handling procedures. Although great care was taken to observe this rule it cannot be excluded completely that quantitative changes observed are partly due to oxidation during handling or electrophoresis. Therefore ascorbic acid was included in some experiments in the electrophoresis buffer as an antioxidant, however, no changes were observed.

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0014-4754/84/090980-03$1.50 + 0.20/0
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Retroviruses released from a human tumor xenograft in nude mice induce colony-stimulating factor (CSF) activity in human fibroblastic cells

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Summary. A human colony-stimulating factor (CSF)-producing tumor transplanted into athymic nude mice released retroviruses in vitro. The viruses induced CSF activity in human fibroblastic cell lines.
Key words. Fibroblastic cells, human; mice, nude; retrovirus; colony-stimulating factor; CSF-producing tumor; tumor xenograft.

Xenotropic infection with murine type C retroviruses is not uncommon in human tumor cells when the tumors have been transplanted into athymic nude mice[1-3]. The tumors inoculated into nude mice and then established as cell cultures in vitro have been shown to release xenotropic murine leukemia virus[1]. It is not clear at present what role, if any, the retrovirus plays in the development of tumors in nude mice by primary human tumor cells. The effects of xenotropic infection of the virus on functional properties of the human tumor cells have not been well investigated.

In 1977, we described a human lung cancer, when transplanted into athymic nude mice, which produced granulopoietic factor(s) (colony-stimulating factor[CSF])[4]. While the patient with this cancer showed only a slight increase in peripheral blood granulocytes, the nude mice into which the tumor was transplanted developed a remarkable granulocytosis. In the plasma of the tumor-bearing mice, and in extracts of the xenografts, high CSF activities were demonstrated. The tumor cells isolated fromt he xenografts also released CSF activity in vitro in culture[5]. While it is not known whether the primary human

tumor, before passage in nude mice, produced CSF in the patient, the discrepancies in the increased granulocyte number between the patient and the tumor-bearing mice are intriguing. On the other hand, murine retroviruses have been shown to induce CSF activities in a variety of rodent fibroblastic cells[6]. This study is an attempt to see whether retroviruses released from this CSF-producing tumor in nude mice induce CSF activities in normal and malignant human cell lines.

Materials and methods. Cell culture. Cells were grown in nutrient medium F-10 supplemented with 10% fetal bovine serum (FBS, Flow Laboratories Inc., Rockville, Md.) at 37°C in an atmosphere of 5% CO_2 in air. Penicillin 100 units/ml and streptomycin 100 µg/ml were added to the medium. Cells in culture and tumors were dispersed with 0.1% trypsin (1:250, Difco, Mich.) solution. The cell line of human male embryo fibroblasts, HT-73 was kindly provided by Dr. M.S. Sasaki (Tokyo Medical and Dental College, Tokyo) and cloned by M. Yasukawa (National Institute of Radiological Sciences, Chiba, Japan). The diploid nature and sex chromosomes of HT-73 cells were determined by Dr T. Ishihara (National Institute of Radiological Sciences). The cell line has characteristics of normal human fibroblasts in vitro, including a limited life span and a well-organized monolayer at low saturation density. The cells did not produce tumors in nude mice when injected s.c. T3M-2 cells have been established from a human leiomyosarcoma of the stomach as described previously[7].

Cultivation of a human lung cancer grown in nude mice (OTUK-tumor). The primary culture of the OTUK-tumor was carried out as described previously[5]. The tumor of 3–6 transfer generations serially passed through nude mice (Balb/C- nu/nu, Central Institute for Experimental Animals, Kawasaki, Japan) was used. The tumor was excised, and cut into small pieces (1–2 mm^3). 10–20 pieces in a plastic petri dish (60 × 15 mm) were incubated with 5 ml of the growth medium (F-10 medium supplemented with 10% FBS) at 37°C in 5% CO_2 in air. Medium from the cultures was harvested at intervals after seeding and centrifuged at 3000 rpm for 20 min. The supernatants collected were frozen at −20°C until used.

Assay for colony-stimulating factor. CSF activity was assayed by a modification of the method described by Bradley and Metcalf[8]. Mouse bone marrow cells were obtained from C3H/HeN strain. They were used after washing with culture medium and removal of erythrocytes by hypotonic lysis. Nonadherent cells were prepared by the method of Messner et al.[9] 1×10^5 of nucleated cells were cultured in a single layer in 1.0 ml of McCoy's 5A medium supplemented with 0.3% agar, 20% FBS, 100 units/ml of penicillin, and 100 µg/ml of streptomycin in the presence of various doses of samples diluted with the medium. After 7 days of incubation, granulocyte and macrophage colonies containing 50 or more cells were counted at 25 × magnification with a dissecting microscope. Individual colonies were aspirated with a micropipette and stained with 0.6% orcein in 60% acetic acid for morphological identification of the colonies. The CSF activity was standardized using the control culture with an aliquot of L-cell conditioned medium containing a known activity as reference.

Assay of reverse transcriptase. Murine leukemia virus (MuLV), was used as the enzyme for reference for the activity. Synthetic copolymer, poly (dT)poly (A) (Miles Laboratories Inc.) was employed as a template. The incorporation of [^{3}H] thymidine triphosphate (sp. act. 26.1–30 Ci/mM, Radiochemical Center, England) was measured as an indication of DNA synthesis. The reaction mixture in a total volume of 0.12 ml contained 4.8 µmol of Tris-HCl (pH 8.1), 0.3 µmol of $MgCl_2$, 3.6 µmol of NaCl, 0.012 µmol of dithithreitol, 2.4×10^{-5} ml of Nonidet P-40, 0.012 µmol of each of dATP, dCTP and dGTP, 6 µCi of [^{3}H] (methyl) TTP, 3 µg of poly (dT)poly (A), 2.16 µg of protein of MuLV as the enzyme and 0.025 ml of fermented broth from actinomyces or basidiomycetes. The reaction mixture was incubated with shaking for 30 min at 37°C. At the end of the incubation, 0.1 ml of the reaction mixture was placed on a round piece of dried filter paper (2.4 cm) presoaked in 0.1 M pyrophosphate solution and each filter paper was immediately dropped into 1.0 ml of cold trichloroacetic acid solution. The fluid was decanted after 10 min and the filter paper was washed twice with cold 5% trichloroacetic acid solution and once with cold 95% ethanol and dried. The tritium radioactivity of the filter paper was measured by a liquid scintil-

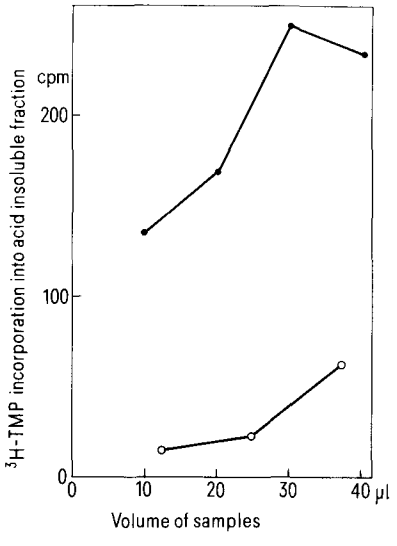

Figure 1. Reverse transcriptase activity in culture media of OTUK-tumor and HT-73 cells treated with retroviruses released from OTUK-tumor. 150 ml of spent medium from the primary culture of OTUK-tumor or from HT-73 cultures treated with 1.5×10^7 virions of the OTUK-derived viruses was used for the assays. HT-73 cells were maintained for 100 days only by medium renewal without passages, and then the spent medium was collected. Retroviruses were concentrated by sucrose gradient ultracentrifugation. The use of synthetic polymer rA.oligo dT under these conditions gave average values of about 5000 cpm per 1×10^9 virions of control murine leukemia viruses. Each point represents a mean value ± SD of 2 determinations. (●——●, OTUK-tumor; ○——○, HT-73 cells 100 days after the treatment with viruses).

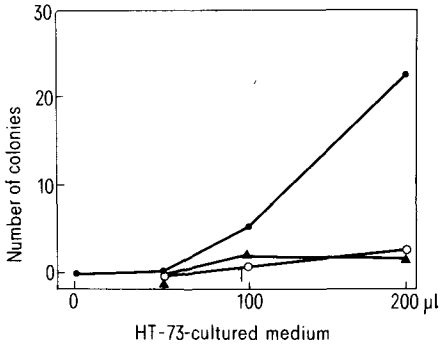

Figure 2. Induction of CSF activities by retroviruses in HT-73 cells. HT-73 cells were treated with the concentrated retroviruses (1.5×10^9 virions) released from the primary culture of OTUK-tumor for an initial 48 h. Then the cultures were washed and the medium was changed every 4 days. On the 20th day of the treatment, the medium harvested was assayed for CSF activity. Each point represents a mean value ± SD for 4 dishes. (●——●, HT-73 cells treated with the concentrated viruses, ○——○, HT-73 cells treated with virus-free supernatant fluids obtained by centrifugation of OTUK-tumor culture medium; ▲——▲, untreated HT-73 cells).

lation counter. The use of the synthetic polymer rA.oligo dT under the conditions described above, gave average values of approximately 5000 cpm per 10^9 virions of control viruses[10].

Results. Release of retroviruses from OTUK-tumor in vitro. In order to concentrate retroviruses, 150 ml of medium from the primary culture of OTUK-tumor, or from HT-73 cells treated with 1.5×10^7 viruses derived from OTUK-tumor medium, was clarified by centrifugation at 3000 rpm for 15 min, respectively. The supernatant fluids were layered over a preformed 15 and 65% (wt/v) discontinuous sucrose gradient prepared in Tris buffer (0.01 M, pH 7.4), and centrifuged in SW 25.2 rotor (Spinco, Beckman) at 25,000 rpm for 1 h. The visible band formed by the virus fraction between the 15 and 65% sucrose layers was removed and dialyzed against Tris buffer for 4–6 h. The final volume of each medium was about 1.4 ml after these procedures. The large and dose-dependent activities of reverse transcriptase found in the concentrated samples from the OTUK-tumor medium are shown in figure 1. The result suggests that the OTUK-tumor releases a large number of retroviruses in vitro in culture.

Induction of CSF activities by the retroviruses. The concentrated viruses (1.5×10^9) prepared from the OTUK-medium were added to confluent cultures of HT-73 cells or T3M-2 cells. After 48 h, the medium was discarded and the dishes were washed twice with F-10 medium, followed by the addition of fresh growth medium. The medium was changed every 4 days and harvested on the 20th day to test for CSF activity. Figure 2 shows that CSF activity was significantly high in the culture medium of HT-73 cells which were treated with the viruses only for initial 48 h. No significant activity was demonstrated in the culture medium of the cells treated with the virus-free supernatant fluids obtained by ultracentrifugation of OTUK-medium. Even if it was not concentrated, the virus-containing supernatant medium (1.5×10^7 virions/ml) from the primary culture of OTUK-tumor could induce CSF activities in HT-73 fibroblasts and T3M-2 sarcoma cells. Figure 3 shows that the supernatant medium, when added to the sarcoma cells, T3M-2, at 1–20% concentrations (v/v) (7.5×10^5–1.5×10^7 virions/dish), induced CSF activities in a dose-dependent manner. To see whether these cell lines produce retroviruses when treated with the viruses culture medium from HT-73 cells treated with 1.5×10^7 virions of the OTUK-derived viruses for the initial 48 h, was harvested after 100 days of the treatment. As described in the preceding section, the viruses were concentrated with discontinuous sucrose gradient centrifugation. HT-73 cells have been shown to release significant amounts of retroviruses even after 100 days of treatment. Reverse transcriptase activities in the culture medium (fig. 1) were demonstrated to be significant, but lower than those of OTUK-medium. HT-73 cells treated with the viruses have been shown to release CSF activities even after 3 transfer generations (data not shown).

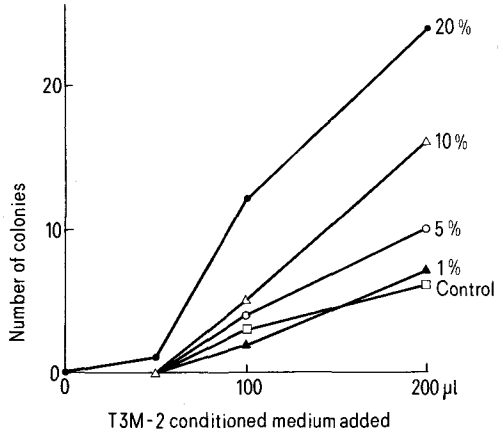

Figure 3. Titration of CSF activities in T3M-2 sarcoma cell line supernatants. T3M-2 cells were treated with the retroviruses from the primary culture of OTUK-tumor. OTUK-culture medium was added to T3M-2 cells at 1, 5, 10 and 20% (vol/vol). On the 20th day, the medium harvested was assayed at various dilutions for CSF activity. 1% of the OTUK-medium contained about 7.5×10^5 virions of the viruses. Each point represents a mean ± SD for 4 dishes. (●——●, T3M-2 cells treated with 20% of OTUK-medium; △——△, 10%; ○——○, 5%; ▲——▲, 1%; □——□, untreated culture).

Discussion. The results show that a human lung cancer transplanted into nude mice releases retroviruses in vitro in culture which induced CSF activities in human normal and malignant cell lines. Xenotropic infection of murine retroviruses in xenografts has been one of the most troublesome problems when we use the nude mouse as a recipient of human tumors[1–3]. CSF activities have been demonstrated in many human tumors transplanted into nude mice which had not produced granulocytosis in the patients themselves[12]. These discrepancies in the increased granulocyte number between the patients and the nude mice bearing the transplanted tumors were also found with the OTUK-tumor, as described previously[4,5]. Together with the results from Koury et al.[6], our observations raise the question whether the tumor cells infected with murine retroviruses induce CSF activities in these tumor cells, which consequently stimulate bone marrow cells to produce granulocytosis in the host mice. It has been known that a transient release of CSF is provoked by some sorts of stimulators, including bacterial toxins, mitogens or nucleotides[13–16]. However, the present findings cannot be explained by a simple stimulation, and probably involved other mechanisms, such as a genetic derangement of cells, since the human fibroblasts treated with the viruses transmitted the CSF-producing function into their progeny. Although we have to rule out the possibility that CSF activity is provided by the virus itself, our study may provide a useful probe for better understanding of the mechanisms by which the expression of CSF genes is controlled.

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