non-hematopoietic tissues. Compared with the distribution of the R4-A9 antigen in adult tissues, wider developmental expression could be detected in other developing tissues during the period of organogenesis. In many instances, the expression of this antigen was restricted to several areas of mesenchyme bounded by an adjacent epithelium, and was not seen in the epithelium. Therefore, this topical expression in the mesenchyme invites speculation that the R4-A9 antigen subserves additional developmental functions.[17,18] In contrast, finally, fetal expression of this antigen that was revealed both in hematopoietic and other developing tissues was lost after birth. Taken together, these results show a marked gradient of R4-A9 antigen expression, with the highest level at the peak of organ development (Table 1). Therefore, the expression pattern observed during this period suggests the possibility that this molecule plays an active role in the control of growth and differentiation in a variety of complex environments containing mesenchymal stromal cells.[18-21]

Little is known about the mechanisms resulting in the appearance of mesenchymal cells expressing R4-A9 antigen during embryogenesis. Interestingly, according to our preliminary experiment on the irradiation of adult mice, mesenchymal stromal cells expressing this antigen were induced in the Peyer's patch and the medullary area of thymus, which show no staining in normal adult mice. In view of the induction of the R4-A9 antigen, it seems likely that its expression in stromal cells is under the direct or indirect influence of soluble factors from nearby tissues. Rigorous testing of this idea in a culture system *in vitro* will be required to analyze the biological function of the R4-A9 antigen.

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# Multipotent Marrow Stromal Cell Line Is Able to Induce Hematopoiesis In Vivo

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Several murine marrow stromal cells were established from murine bone marrow cultures. Stromal cell lines transfected with a tumor-inducing polyoma virus middle T antigen (MTAg) were inoculated into nude mice subcutaneously. KUSA-MTAg cells, one of these cell lines, led to the rapid local development of bone marrow consisting of trilineage hematopoietic cells and bone; other cell lines produced spindle cell sarcoma or hemangiosarcoma. These results suggested that a single stromal cell line, KUSA-MTAg cells, may induce hematopoietic stem cells or early progenitors of three lineages of hematopoietic cells in vivo. Interestingly, untransfected KUSA cells expressed three new mesenchymal phenotypes, osteocytes, adipocytes, and myotubes, after treatment with 5-azacytidine.


Bone marrow stromal cells are thought to be a prerequisite for the proliferation of multipotent stem cells. The growth and differentiation of hematopoietic stem cells have been achieved successfully in Dexter-type, long-term bone marrow cultures (LTBMCs) in vitro (Dexter et al., 1977; Greenberger et al., 1979; Dexter and Shadduck, 1980). Cells with lymphoid potential could also be grown efficiently in modified marrow culture (Whitlock and Witte, 1982). Cells that grow under these conditions are almost entirely pre-B cells that have undergone rearrangement for the immunoglobulin heavy chain but not the light chain gene (Whitlock and Witte, 1987). These studies demonstrate that various hemopoietic cell lineages can be grown in marrow culture under the appropriate conditions.

Marrow stromal cells seem to communicate with hematopoietic cells through cell-to-cell signals from colony-stimulating factors (CSFs) (Gough et al., 1984; Metcalf, 1985, 1986; Tuchiya et al., 1986; Rajavashisth et al., 1987; Ladner et al., 1988), membrane-bound molecules (Kodama et al., 1982; Zipori et al., 1985), extracellular matrix (Roberts et al., 1988), and gap junctions (Watanabe, 1985; Yamazaki et al., 1989). Among these, the humoral factors have been mainly characterized. CSF-1, which stimulates the differentiation of certain hematopoietic stem cells or leukemic blast cells into a monocytic lineage (Stanley et al., 1978; Guilbert and Stanley, 1980; Stanley et al., 1983; Miyauchi et al., 1988a, b), was found to be produced constitutively by most of the stromal cell clones, such as ALC-5, 30E, D2XRII, and H-1 (Harigaya et al., 1981; Greenberger et al., 1984; Hunt et al., 1987). CSF-1 is also detected in supernatant from LTBMCs using a sensitive radioimmunoassay (Shadduck et al., 1983), but the addition of exogenous purified CSF-1 or an antibody to CSF-1 has only limited effects on the proliferation of hematopoietic cells in LTBMCs (Dexter and Shadduck, 1980). The GY-30 cell line can respond to external signals such as interleukin-1 and lipopolysaccharide by inducing the production of G-CSF and GM-CSF at the mRNA level (Rennick et al., 1987). Certain stromal cells and 3T3 cells have been reported to maintain colony-forming unit spleen cells (Till and McCulloch, 1961; Kodama et al., 1982; Zipori et al., 1985; Roberts et al., 1987) and early lymphoid and myeloid cells in vitro (Hunt et al., 1987).

Knospe et al. (1986, 1989) reported that in vivo hematopoiesis was simulated by noncellular-matrix-deposited membranes implanted intraperitoneally or subcutaneously. Crude extract from osteosarcoma cells is reported to elicit hematopoiesis in the subcutaneous tissue of adolescent mice (Takaoka, 1985). In the present study, we generated a single cloned immortalized stromal cell, KUSA, which induces a tumor in Balb/c nude mice and trilineal hematopoiesis in the tumor when inoculated subcutaneously. Other stromal cell lines did not support hematopoietic cell development.

## MATERIALS AND METHODS

### Cell culture

Dexter LTBMCs derived from the bone marrow of female C3H/He mice were established as described (Dexter et al., 1977). In week 14 of culture, the adherent cells were removed with trypsin/EDTA (GIBCO) and transferred into two flasks. After persistent feeding of these cultures for 4 to 6 weeks, the cultured cells became homogeneous and were devoid of hematopoietic cells. The cultures were then passaged, and the cell

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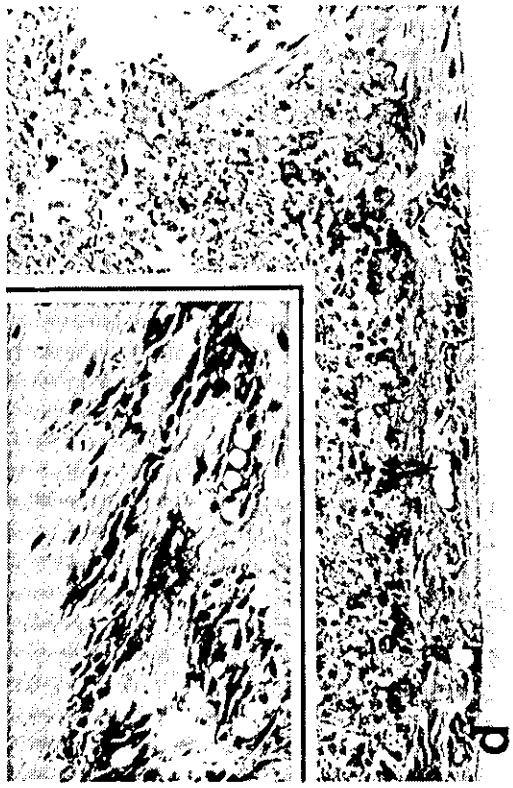
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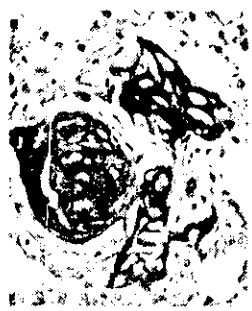


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TABLE 1. Hematopoiesis induced by marrow stromal cells and subclones of KUSA cells[1]

(a) Marrow stromal cells

| Cells | Hematopoiesis EMG[2] | EG[3] | G[4] | Tumor |
|---|---|---|---|---|
| KUSA-MTAg | 4/8 | 3/8 | 0/8 | 8/8 |
| KUSA-MAS | 5/6 | 1/6 | 0/6 | 6/6 |
| KUSA-NEO and KUSA | 2/4 | 1/4 | 1/4 | 4/4 |
| KUM3-MTAg | 0/6 | 0/6 | 0/6 | 6/6 |
| KUM4-MTAg | 0/6 | 0/6 | 0/6 | 6/12 |
| F2 | 0/5 | 0/5 | 0/5 | 5/5 |
| NIH 3T3/MTAg | 0/5 | 0/5 | 0/5 | 5/5 |

(b) Subclones of KUSA cells

| Cells | EMG[2] | EG[3] | G[4] | Tumor |
|---|---|---|---|---|
| KUSA/A1 | 12/12 | 0/12 | 0/12 | 12/12 |
| KUSA/M1 | 6/6 | 0/6 | 0/6 | 6/6 |
| KUSA/D | 3/3 | 0/3 | 0/3 | 3/3 |
| KUSA/H1 | — | — | — | 0/4 |
| KUSA/O | — | — | — | 0/3 |

[1] $10^7$ cells in a volume of 0.4 ml were injected subcutaneously into female Balb/c nu/nu nude mice, 3-6 weeks of age. Tumors were excised 3-8 weeks after injection and fixed with 20% formalin. The tumor was embedded in paraffin, cut into sections, and stained with hematoxylin and eosin or von Kossa.
[2] Trilineage hematopoietic cells, i.e., erythrocytic, granulocytic, and megakaryocytic lineage cells, were observed.
[3] Bilineage cells, i.e., erythrocytic and granulocytic lineage cells, were seen in the tumor.
[4] Only granulocytic lineage cells were seen.
These hematopoietic cells were determined morphologically and histochemically (see Fig. 1). Few lymphocytic cells were observed in the tumors.

lines obtained have since been maintained for 4 months and subcultured twice weekly.

Cell lines from different dishes were subcloned by limiting dilution. The cells were then treated with trypsin/EDTA, and $10^2$-$10^3$ marrow stromal cells were dispersed for cloning on 100 mm plates in Iscove's medium supplemented with 20% fetal bovine serum. Eight different cell lines were obtained. Subclones were amplified by successive cultures in liquid medium. Stock cell pellets were frozen in glycerol medium in liquid nitrogen.

Cells from logarithmically growing stock cultures were seeded into 60 mm culture dishes at $10^3$ cells per dish. In order to see cell differentiation, cultures were treated with 1, 10, or 50 μM of 5-azacytidine (Sigma Chemical, St. Louis) for 24 hours. 5-azacytidine is a cytidine analog capable of altering the expression of certain genes, some of which may control the program for differentiation (Constantinides et al., 1978, Taylor et al., 1979, Okada et al., 1984). The cultures were subsequently fed twice weekly until the experiment was terminated.

Fig. 1. Microscopic examination of hematopoiesis (trilineage hematopoietic cell proliferation) induced by KUSA MTAg cells. (a) Hematopoiesis induced by KUSA-MTAg cells. Four weeks after inoculation of $10^7$ KUSA-MTAg cells subcutaneously, the tumors were excised. Complete functional hematopoiesis was seen with ectopic new bone formation in the tumor. Hematoxylin and eosin (H.E.) stain. ×60. (b) Higher magnifications of hematopoietic cells seen in the tumor. H.E. stain. ×200. Granulocytic lineage cells, erythrocytic lineage cells, megakaryocytes (arrowheads) were seen. These cells were identified mainly by H.E. staining and confirmed by enzyme histochemistry of acetylcholine esterase (megakaryocytes) and immunohistochemistry using hemoglobin F antibody (erythrocytic lineage).(c) Bone formation by KUSA-MTAg cells was observed in lung. ×64. inset: Higher magnification of bone. Mallory stain. ×200. (d) In vivo diffusion chamber cultures of KUSA-MTAg cells. H.E. stain. ×100. A diffusion chamber containing KUSA-MTAg cells was implanted subcutaneously. The chamber was removed, fixed and demineralized 4 weeks after implantation. No hematopoiesis was observed in the diffusion chamber culture, although bone and marrow cavity were formed. inset: Higher magnification. H.E. stain. ×200.

### Transfection of polyoma middle T gene (MTAg) into cells

The cDNA clone of polyoma virus MTAg was linked with mouse mammary tumor virus (MMTV) LTR (Lee et al., 1981). Then, the plasmid, pMMMTAg, was ligated with the DNA fragment (1,400 bp) containing the gene for conferring G418 resistance (Ono et al., 1988). The resulting plasmid, pMMMTAg-neo, was transfected by the method of Graham and Van Der Eb (1973) to stromal cells. Twenty-four hours after transfection, selection media containing 200 μg/ml of G418 were added. G418 resistant colonies were analyzed for hematopoietic induction in vivo.

### Cell transplantation

Freshly scraped confluent cells ($10^7$) were injected subcutaneously into Balb/c nu/nu mice (Sankyo Laboratory, Hamamatsu, Japan). Animals were sacrificed by cervical dislocation between 3 and 8 weeks after inoculation.

### In vivo culture in diffusion chamber

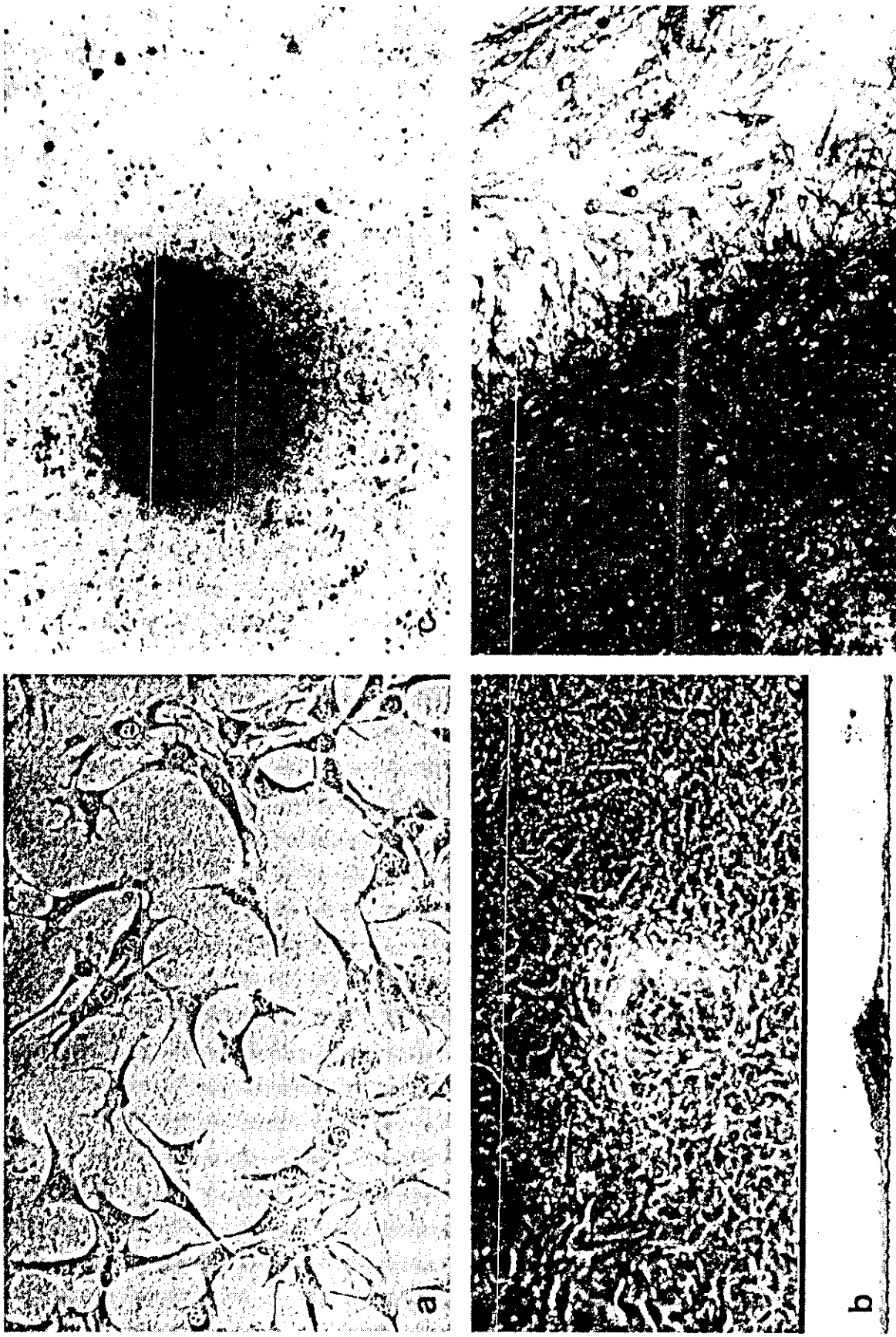
Paired diffusion chambers (Urist et al., 1982) were constructed using lucid rings and 0.45-μm membrane filters (Millipore, Bedford, MA). The chambers shared a common filter. One chamber contained KUSA-MTAg cells in 200-μl aliquots from 6 dishes of confluent cultures. Eight diffusion chambers and their contents were implanted subcutaneously in 8–12-week-old female C3H/He mice, one chamber per mouse. The animals were sacrificed after 4 to 8 weeks. The chambers were fixed in 20% formalin and demineralized. Serial paraffin sections were prepared and stained by either hematoxylin and eosin or Mallory staining.

### Cytochemical staining for alkaline phosphatase activity

Cells in 35 mm dishes were maintained in the standard media described above. Fixed cultures were stained for 30 minutes at 37°C using naphtol AS-MX phosphate as a substrate and fast red violet LB salt as a coupler. Some cultures were maintained with standard media supplemented with 50 μg/ml ascorbic acid and 10 mM sodium β-glycerophosphate for up to 30 days in vitro. After in situ fixation, the cultures were stained using the von Kossa method (von Kossa, 1901) or Oil Red O method (Lillie, 1943).

### RNA blot analysis

RNA was prepared from cultured cells by homogenization in guanidinium isothiocyanate, followed by centrifugation over a cesium chloride cushion (Chirgwin et al., 1979). The RNA was electrophoresed on 1.0% agarose gel, transferred to a nitrocellulose filter (Du Pont Company NEN Products), and hybridized with labeled cDNA inserts as indicated below for 14–16 hours in buffer containing 5 × Denhardt's solution, 0.02% poly A, and 1% sodium dodecyl sulfate (SDS) at 65°C. The blots were washed with 2 × SSC (1 × SSC = 0.15 M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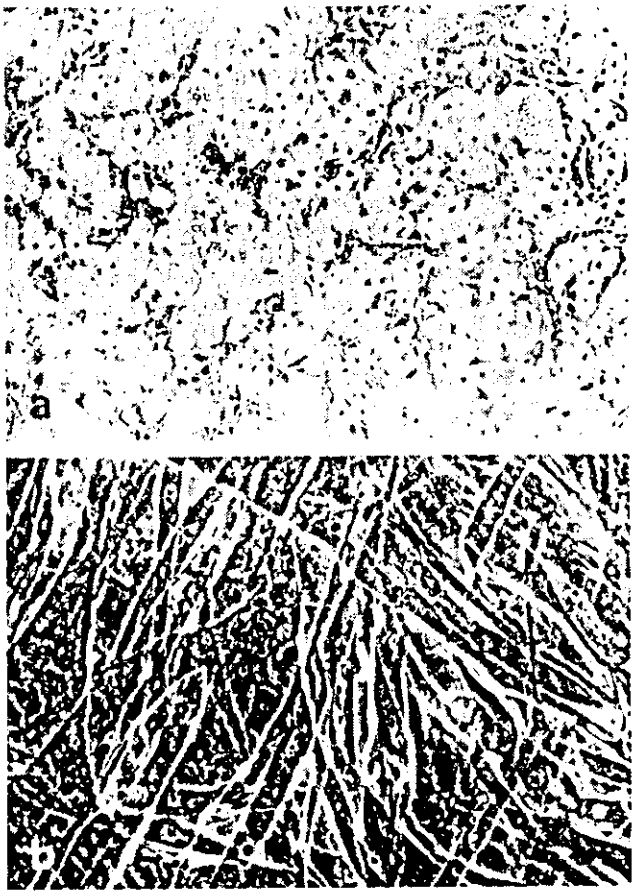

Fig. 3. Adipocyte and myogenic differentiation of KUSA cells. Semiconfluent KUSA cells were treated with 50 mM of 5-azacytidine for 24 hours. Adipocytes (a), bone nodule, and myotubes (b) were seen 28 days after treatment. These cells usually appeared in clusters or foci. Multinucleated myotubes were capable of contraction without stimulation. The contraction and relaxation continued at least 1 week. Certain myotubes were striated. Fat droplets of KUSA adipocytes were stained positively with Oil-Red O. Phase-contrast field. ×400.

NaCl, 50 mM $NaH_2PO_4$, 5 mM EDTA) containing 1% SDS at room temperature and 65°C. Final washing was carried out with 0.1 × SSC containing 0.1% SDS at 65°C for 30 minutes. The CSF-1 probe was a 3.9 kb Eco RI fragment from pGEM2MCSF-10, kindly provided by Dr. Price (Cetus Co., Emeryville, CA), which was cloned from mouse L cells (Ladner et al., 1988). The G-CSF probe was a 1 kb Bam HI-Eco RI fragment from pMG-3 (a gift from Dr. Nagata) (Tuchiya et al., 1986). The GM-CSF probe was a 0.75 kb Bam HI-Eco RI fragment from pGMG 3.2 (a gift from Dr. Dunn) (Gough et al., 1984). The mast cell growth factor probe was a 2.06 kb SalI fragment from pBSSK:MGF10.1 (a gift from Dr. Gillis) (Anderson et al., 1990).

Fig. 2. Phase contrast micrograph of KUSA cells at the semiconfluent and confluent stage, light micrograph of nodule formation, and cytochemistry of ALP activity. (a) KUSA cells at semiconfluent stage. Phase contrast field. ×400. (b) Nodule present in a KUSA culture 14 days postconfluence. Phase contrast field. ×400. Lower photo shows a vertically sectioned nodule. KUSA cells were arranged at the periphery of the nodule, which was enveloped by a periosteum-like cell layer. Bone nodules consisted of an eosinophilic matrix containing ovoid cells resembling osteocytes. Methylene blue stain. ×100. (c) Nodule present in a KUSA culture 30 days postconfluence. KUSA culture in supplemented medium containing 10 mM β-glycerophosphate was fixed and stained in situ by von Kossa technique. Discrete mineralized nodules are present. These were characterized as bone (Sudo et al., 1983). ×100. (d) Cytochemistry of alkaline-phosphatase activity. The cells were strongly reactive for alkaline-phosphatase activity. ×100.

### Bone marrow colony-forming assay

CFUc were allowed to proliferate in semisolid, soft agar cultures as described by Pike and Robinson (1976). Fresh murine bone marrow cells were obtained from 8- to 12-week-old female C57/BL mice by flushing the tibias and femurs. Fresh marrow cells were inoculated in 1 ml of complete Iscove's medium containing 0.3% agar (Difco Laboratories, Detroit) and 10% conditioned media in plastic Petri dishes. The conditioned medium was harvested from KUSA-MTAg cells grown for 3 days in Fisher's medium supplemented with 10% fetal bovine serum (FBS). After incubation for 8 days at 37°C in an atmosphere of 7.5% $CO_2$ in humidified air, the specimens were air-dried and stained by a double-staining technique using naphthol-ASD-chloroacetate and α-naphthyl butyrate as substrates (Li et al., 1973).

In another series of experiments, hematopoietic cells were obtained from the 10 tumor samples induced by KUSA-MTAg cells 30 days after injection by flushing the tumor. The hematopoietic cells were plated at $2.5 \times 10^4$ cells/dish of 0.3% agar in Iscove's medium supplemented with 10% FBS. Colony formation was stimulated by the addition of WEHI-3 conditioned medium. The staining of specimens was carried out as described above. The mean colony number was calculated from the results of 40 culture dishes.

## Results

Eight stromal cell lines were isolated from murine LTBMCs. These clonal cells were designated KUSA, KUM 1, 3 to 7, and 9. All the clonal cells were fibrocytic in appearance. The plasmid containing middle T antigen (MTAg) gene from polyoma virus was transfected to the stromal cell lines. After selection with G418, MTAg transfected KUSA, KUM 3, and KUM 4 cells (KUSA-MTAg, KUM 3-MTAg, and KUM 4-MTAg, respectively) were isolated. These three cells were tumorigenic when inoculated into nude mice.

Three to 8 weeks after the inoculation of cells, tumors were excised to see if stromal cells stimulated hematopoiesis in vivo. Tumors were formed by KUSA-MTAg, KUM 3-MTAg, and KUM 4-MTAg cells. Four weeks after the injection of KUSA-MTAg cells, bone formation was observed around the periphery of the tumor (Fig. 1a). Hematopoiesis was observed inside the bony capsule, as is the case in bone marrow in situ (Fig. 1a,b, Table 1). Trilineage hematopoietic cells, i.e., granulocytic, erythroblastic, and megakaryocytic cells, were recognized in the tumor, as determined by their morphology, and staining with naphthol AS-D chloroacetate esterase, peroxidase, acetylcholinesterase, and pan-leukocyte antigen. When a number of histological sections were examined, no mitotic cells were present in the stromal cells in vivo, suggesting that most of

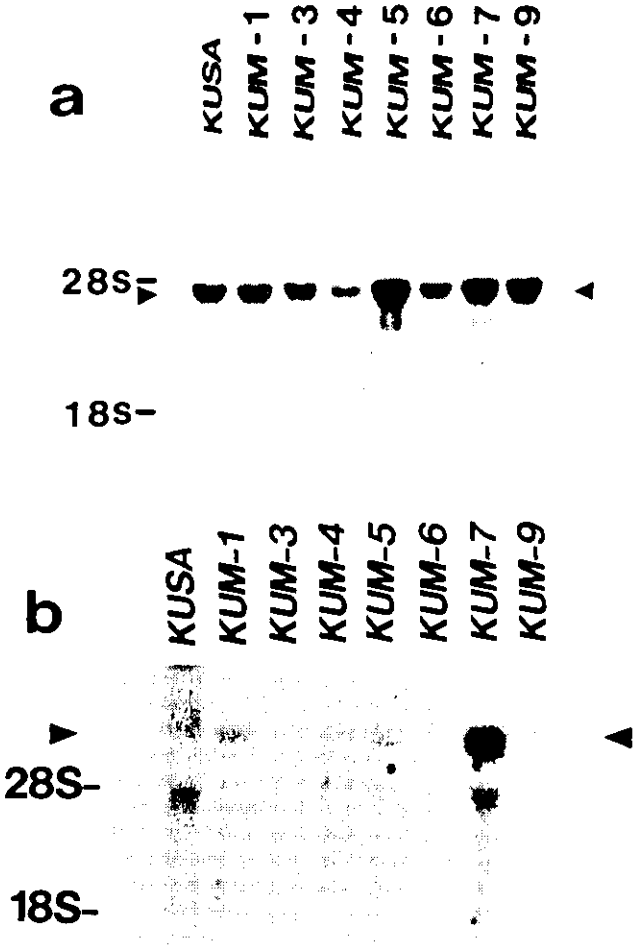


Fig. 4. Hybridization analysis of CSF-1 (a) and MGF (b) of KUSA cells and other stromal cell lines. RNAs were extracted from each cell line at confluence; 5 μg of total RNAs were electrophoresed on each lane. The blot was hybridized with the CSF-1 probe or the MGF probe at 65°C for 16 hours. Then the blot was washed with 2% SDS, 2X SSC solution twice at 65°C for 30 minutes. Final washing was carried out with 0.1 × SSC containing 0.1% SDS at 65°C for 30 minutes. The exposure was carried out for 48 hours at −80°C, using an intensifying screen (a and b). The positions of the CSF-1 (a) or MGF (b) signal and 28S and 18S markers are indicated.

these cells were not dividing. Thus tumors induced by KUSA-MTAg cells appeared to result from the rapid migration of hematopoietic cells into the transplanted cultured cells and growth of such cells rather than the neoplastic proliferation of the stromal cell lines.

To determine whether the hematopoiesis was caused by the original function of the stromal cell per se, similar experiments were done using F2, NIH-MTAg, KUSA-MAS, and KUSA-NEO cells. KUSA-MAS and KUSA-neo cells were transfected with the c-terminal portion of middle T antigen gene (MAS) and the gene conferring G418 resistance, respectively. Both cells induced hematopoiesis similar to that observed with the KUSA-MTAg cells. Other stromal cell lines or fibrocytic cell lines did not induce hematopoiesis in vivo, although cells were inoculated into nude mice in the same way (Table 1a). F2 and NIH-MTAg cells were both NIH-3T3 cells transfected with the middle T antigen gene. The promoters for transfection of the MTAg gene are MMTV-LTR and polyoma virus early promoter, respectively. Neither F2 nor NIH-MTAg cells induced hematopoiesis in vivo but did form localized fibrocytic sarcomas. KUM 4 cells had the ability to differentiate into adipocytes, irrespective of the presence of MTAg. KUM 3 cells did not show any differentiated phenotypes in vitro. Although KUM 3 and KUM 4 are of marrow stroma origin, neither elicited hematopoiesis. They generated localized tumor of fibrocytic sarcomas and angiosarcomas, respectively.

Subclones of untransfected KUSA cells were isolated to assess the clonality of the cells and were injected subcutaneously in the same way as KUSA-MTAg cells (Table 1b). KUSA/A1, KUSA/M1, and KUSA/D cells elicited trilineage hematopoiesis again, whereas KUSA/H1 and KUSA/O did not produce tumors or hematopoietic cells. To determine whether KUSA-MTAg cells induced hematopoiesis in other organs as well as in the subcutaneous tissue, these cells were injected into the tail vein. When KUSA-MTAg cells were injected intravenously, multiple areas of bone formation were observed in lung (Fig. 1c). However, the cells did not induce hematopoiesis.

From these results, we assume that KUSA-MTAg cells support hematopoiesis by providing an environment for circulating hematopoietic progenitor cells. An alternative hypothesis is that KUSA-MTAg cells give rise to hematopoietic cells through differentiation. To examine these hypotheses, we carried out in vivo diffusion chamber cultures of KUSA-MTAg cells so that cells of recipient mice origin could not migrate into the KUSA-MTAg cell tumor. If KUSA-MTAg cells merely provide a suitable environment, no hematopoiesis would be seen in the diffusion chamber culture. However, if KUSA-MTAg cells themselves differentiate into hematopoietic cells, hematopoiesis would be seen, as is the case with direct injection of KUSA-MTAg cells. Eight in vivo diffusion chamber cultures of KUSA-MTAg cells were implanted subcutaneously. None of the cultures exhibited hematopoiesis 4 to 8 weeks after implantation, although cells exhibited marrow cavity and osteogenesis (Fig. 1d). Therefore, we concluded that the hematopoietic cells in the tumors were of recipient mice origin.

Morphologically, the tumor resulted from the subcutaneous injection of KUSA-MTAg cells contained immature hematopoietic cells. To determine whether hematopoietic progenitor cells were present in the tumor, we performed a CFU-c assay on the hematopoietic elements in the tumor. The number of CFU-c per $2.5 \times 10^4$ cells was $13.7 \pm 9.0$ (mean ± standard deviation) for 10 tumor samples. The result confirmed that KUSA-MTAg cells support primitive hematopoietic cells.

Untransfected KUSA cells in logarithmic growth phase showed a fibroblastic morphology (Fig. 2a). After confluence, the cellular outlines had a mosaic appearance. Small nodular regions were identified 2 to 4 weeks after confluence (Fig. 2b). Bone nodules were stained positively with von Kossa staining in a medium containing β-glycerophosphate (Fig. 2c). When stained in situ by the von Kossa method, they appeared as black (mineralized) discrete nodules. Cytochemical

TABLE 2. Production of colony-stimulating activity by KUSA cells and inhibition by an antibody directed against CSF-1[1]

| Concentration of antibody | Concentration of NRS[2] | Number of target cells $0.8 \times 10^4$ | Number of target cells $2.0 \times 10^4$ |
|---|---|---|---|
| 1:256 | | 0.0 | 0.0 |
| 1:512 | | 0.0 | 0.0 |
| 1:1,025 | | 0.0 | 0.0 |
| | 1:256 | 36.5 ± 10.0 | 49.8 ± 4.3 |
| | 1:512 | 30.0 ± 7.5 | 53.8 ± 5.4 |
| | 1:1,024 | 28.8 ± 7.2 | 60.3 ± 14.4 |
| Control with KUSA cell conditioned medium | | 21.5 ± 2.1 | 53.8 ± 6.1 |

[1]Medium was harvested from KUSA cells grown for 3 days in Fisher's medium supplemented with 10% fetal bovine serum and antibiotics. Fresh marrow cells were inoculated in 1 ml of complete Iscove's medium containing 0.3% agar and 10% conditioned media in plastic Petri dishes. On the eighth day, colonies consisting of 50 or more cells were counted. All the colonies were picked, applied to glass slides, and analyzed after staining fixed cells with a double-staining technique using naphthol-ASD-chloroacetate and α-naphthyl butyrate as substrates. Results of four independent experiments were pooled and presented as the mean percent of number of colonies.
[2]Normal rabbit serum.

staining for alkaline phosphatase activity was also performed. KUSA cells showed extremely high activity (Fig. 2d). These characteristics of the cells were consistent with those of osteogenic cells (Sudo et al., 1983).

To determine the ability of untransfected KUSA cells to differentiate, they were exposed to 5-azacytidine for 24 hours (Constantinides et al., 1978). After the removal of the analog, there was no subsequent treatment with 5-azacytidine. Bone nodules appeared on day 20 after treatment, as in the untreated condition. On day 24 after treatment, cells began to accumulate small fat droplets, which were stained with Oil Red O in the cytoplasm (Fig. 3a). Four weeks after treatment, cells differentiated into multinucleated muscle cells, which showed striation in their cytoplasm (Fig. 3b). Surprisingly, KUSA cells that differentiated into muscle cells can repeat contraction and relaxation without any stimulation. The contraction of KUSA cells is rapid and automatic, completely different from that of mesangial smooth muscle cell in vitro (Venkatachalam, 1985).

To examine CSF expression in KUSA cells as well as other cell lines, blot hybridization of KUSA cell RNA was done with probes of mouse CSF-1, GM-CSF, G-CSF, and mast cell growth factor (MGF). Distinct mRNA band of 4.5 kb in size was detected in KUSA cells as well as other stromal cells with a cDNA probes for CSF-1 (Fig. 4a), whereas no bands were observed with probes for GM-CSF and G-CSF (data not shown). MGF RNA was expressed in KUSA cells; however, the level of expression was low (Fig. 4b). Production of CSF by KUSA cells was also analyzed using a colony bioassay (Table 2). Colonies formed in cultures containing 10% KUSA cell-conditioned medium were mainly diffuse macrophage-like colonies. This colony stimulating activity was inhibited completely by an antibody directed against murine L cell CSF (Shadduck et al., 1979).

## DISCUSSION

Certain cell lines are known to support hematopoietic stem cells, e.g., CFU-s or early progenitors of hematopoietic cells, in vitro (Kodama et al., 1982, Zipori et al., 1985). The experiments in this report were designed to determine whether hematopoiesis was induced only by a single stromal cell line in vivo. The previous study using H-1 cells failed to show in vivo hematopoiesis (Harigaya and Nakamura, 1985). In the present study, we showed that KUSA-MTAg cells as well as untransfected KUSA cells induced hematopoiesis in subcutaneous tissue. The induced hematopoiesis was not incidental, since trilineage or bilineage hematopoietic cell proliferation was observed in most of the tumors. Further, KUSA-MTAg cells elicited hematopoiesis specifically, since other stromal cell lines did not share this property. Although the KUSA cell line was cloned by limiting dilution, this line was subcloned in order to assure the clonality of cells. All the subclones of KUSA cells that formed tumors induced trilineage hematopoietic cell proliferation. Therefore, it appears that a single stromal cell line is capable of eliciting hematopoiesis in vivo.

The expression of polyoma MTAg provides a means to transform fibroblastic cells to confer tumorgenic activity in nude mice (Rassoulzadegan et al., 1982). The expression of polyoma MTAg gave rise to the nature of the cell itself, that is, endothelioma cells can form hemangioma; embryonal cells, teratocarcinoma, fibroblast cells, sarcoma (Williums et al., 1989). Therefore, hematopoiesis is induced by the direct or indirect function of KUSA cells as a marrow stromal cell per se rather than the activity modulated by polyoma MTAg. In contrast, transfection of oncogenic virus simian-virus-40 alters the growth and differentiated properties of human epidermal keratinocytes (Steinberg et al., 1979; Defendi et al., 1982).

How does hematopoiesis occur? One of the most striking aspects of the hematopoiesis described here is the speed with which it took place in the host animal. Within a week, KUSA cells formed a tumor subcutaneously. Within 3 weeks, hematopoiesis was observed in the tumor. The most fascinating hypothesis concerning hematopoiesis is that KUSA cells have the ability to attract or home hematopoietic stem cells or early progenitors of trilineage cells. Alternatively, KUSA cells can induce stromal cells to produce a microenvironment suitable for the growth of both bone and trilineage hematopoiesis in subcutaneous tissues. Knospe et al. (1989) reported that noncellular matrix factors laid down on cellulose ester membrane by bone can induce a microenvironment suitable for trilineage he-

matopoiesis in subcutaneous tissues. Thus bone formation may be necessary for the induction of hematopoiesis. However, it is obvious that bone formation by cells is not sufficient for hematopoiesis, since most osteogenic cells do not generate hematopoiesis in vivo (Majeska et al., 1980; Partridge et al., 1981). KUSA cells are capable of differentiating into adipocytes, osteocytes, and myogenic cells, similar to 10T1/2 cells (Taylor et al., 1979). Since stromal preadipocytes are reported to be important for the proliferation of multipotent stem cells (Dexter et al., 1977, Kodama et al., 1982, Zipori et al., 1985), the ability of KUSA cells to differentiate into adipocytes is interesting. Cooperation between KUSA adipocytes and osteocytes may be necessary for the induction of hematopoiesis in vivo, because another marrow stromal cell, H-1/A, which differentiates into adipocytes, does not induce hematopoiesis in vivo (Harigaya and Nakamura, 1985).

KUSA cells as well as many other cell lines express CSF-1 and MGF constitutively (Greenberger et al., 1984; Hunt et al., 1987; Anderson et al., 1990; Umezawa et al., 1990), but not GM-CSF and G-CSF at detectable mRNA levels. The colony stimulating activity of KUSA cells was completely inhibited by an antibody to L-cell CSF. KUM 3, KUM 4, and H-1/A, which express CSF-1 (Umezawa et al., 1991), and KUM 4 and NIH-3T3 cells, which express MGF (Dolci et al., 1919), did not induce hematopoiesis as a result of subcutaneous injection. Therefore, the known colony-stimulating factors produced by KUSA cells were of no consequence for the induction of trilineage hematopoiesis. It remains, however, to be seen whether new or as yet unknown growth factors or membrane-bound molecules may be involved in the cell-to-cell communication between stromal cells and multipotent hematopoietic stem cells in vivo (Torok-Storb, 1988).

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CELL STRUCTURE AND FUNCTION 17: 177-184 (1992)


# Expression of Gap-Junctional Protein (Connexin 43 or α1 Gap Junction) is Down-Regulated at the Transcriptional Level during Adipocyte Differentiation of H-1/A Marrow Stromal Cells

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Key words: gap junction/adipocyte/bone marrow/differentiation/stroma cell

*ABSTRACT.* **Bone marrow stromal cells are requisite for the proliferation of hematopoietic cells and communicate with each other via gap junctions. Marrow stromal cells expressed connexin 43, but not connexin 32. H-1/A, a murine marrow stromal cell line, underwent adipocyte differentiation at confluence, and expressed the 3.0 kilobase mRNA species of connexin 43 before differentiation. H-1/A cells studied with the dye-transfer method showed gap-junctional communication with adjacent cells but lost this communication during differentiation. The connexin 43 transcripts in H-1/A cells were down-regulated before the expression of glycero-monophosphate dehydrogenase was induced. Loss of gap-junctional communication was regulated at the mRNA level of connexin 43. Connexin 43 expression was down-regulated at the transcriptional level.**

Gap junctions between cells are present in many tissues and in a wide range of species. Gap junctions are composed of protein channels that allow molecules with molecular weights of less than 1500 to pass directly from the inside of one cell to that of another (16, 17, 21). Cells connected by gap junctions share many of their small molecules, hence gap junctions are clearly important in coordinating the activity of these cells and expressing a cell's individuality. cDNA for mammalian gap-junction proteins has been cloned, and it appears that there are tissue-specific gap-junctions (4, 19, 24, 39). The three Xenopus gap junction cDNAs have also been isolated and found to be expressed differentially during the development of Xenopus embryos (10, 11). Gap junctions have also been detected between mammalian marrow stromal cells *in vivo* by means of electron microscopy (36). *In vitro*, gap junctions have been observed in bone marrow cultures and cell lines by means of electron microscopy (2, 6, 37, 38) and the dye-transfer method (30, 31).

Intercellular communication via gap junctions is reported to be correlated with early embryogenesis, terminal differentiation and cell growth (3, 18, 35). 3T3-L1 cells lose virtually all cellular communication when induced to differentiate into adipocytes (3). The induction of cell growth causes a transient reduction in communication in 3T3-L1 cells and is related to the tumor-promoting phorbol ester-induced modulation of cell-to-cell and cell-to-substratum interactions (25, 26, 27). Meanwhile, a marrow stromal cell line, H-1/A, cloned from the bone marrow of C57/Bl mice spontaneously differentiates into adipocytes *in vitro* (13) and expresses a marrow stroma-specific antigen (1). H-1/A cells contribute to granulopoiesis during the fibrocytic stage by producing colony-stimulating factor 1 (CSF-1) (23, 32). With the dye transfer method, H-1/A cells *in vitro* were found to communicate with each other via gap junction (30, 31). Gap-junctional communication of H-1/A cells also decreases during adipocyte differentiation as in 3T3-L1 cells. However, the mechanism regulating loss of communication remains unknown. This study reports that the loss of gap-junctional communication is controlled at a connexin 43 transcriptional level and precedes the switching-on of the glycero-monophosphate dehydrogenase (GPD) gene during adipocyte differentiation of H-1/A stromal cells.

## MATERIALS AND METHODS

*Cell culture.* H-1/A cells were routinely cultured with Fischer's medium (GIBCO, Grand Island, N.Y.) supplemented with 10% horse serum (Irvine Sci., Santa Ana, Calif.), penicillin, and streptomycin. The cultures were incubated at 33°C in an atmosphere of 5% carbon dioxide. In certain cases, H-1/A cells were also treated with 200 U/ml or 1,000 U/ml of cachectin/TNF (Genzyme Co., Boston, MA) at confluence and 6 days after confluence. To estimate adipocyte differentiation, dishes were fixed with 10% buffered neu-

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tral formalin and stained with 0.5% Oil Red O in propylene glycol.

*RNA blot analysis.* Fresh unsupplemented medium, or medium containing 200 U/ml or 1,000 U/ml of cachectin /TNF was added to semiconfluent or confluent layers of H-1/A cells. After incubation for 12, 24, or 72 hours, cells were harvested for RNA blot analysis. RNA was prepared from cultured cells by homogenization in guanidinium isothiocyanate, followed by centrifugation over a cesium chloride cushion (5). The RNA was then electrophoresed in a 1.0% agarose gel, transferred to a nylon filter (Du Pont Company NEN Products), and hybridized with cDNA inserts labeled with $^{32}$P-dCTP by the random-primer method (9) at 65°C for 14-16 hours in buffer containing 5×SSPE [1×SSPE is 0.15 M NaCl, 10 mM $NaH_2PO_4/Na_2HPO_4$ (pH 7.4), 1 mM EDTA], 5×Denhardt's solution (1×Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 0.02% poly(A), and 1% SDS. The blots were washed with 2×SSC (1×SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.4) containing 1% SDS at room temperature and 65°C. Final washings were performed with 0.1×SSC containing 0.1% SDS at 65°C. The blots were exposed to X-ray film at −80°C using an intensifying screen.

The connexin 43 was a 1.4 kilobase (kb) EcoRI fragment from G2A (4). The connexin32 was a 1.5 kb EcoRI fragment (24). The GPD probe was a 0.9 kb EcoRI fragment of pL10.9 (28). The actin probe was a 6.8 kb EcoRI fragment of pSP62-PL (15). With the use of this α-actin DNA, β-actin mRNA was detected by RNA blot analysis to check the total amount of RNA loaded. Quantification of RNA hybridized to each probe was determined by densitometry of bands using Quick Scan R & D (Helena Laboratories).

*Communication assay.* Intercellular transfer of fluorescent Lucifer Yellow CH (Sigma, ST Louis, USA) was measured after direct microinjection of the dye into a cell under a phase-contrast microscope and observation of its transfer to neighboring cells under a fluorescence microscope. Microinjection was carried out using a micromanipulator (Narishige Co., Tokyo, Japan). A 10% solution of Lucifer Yellow CH in a 0.3 M lithium chloride solution was transferred to a glass capillary needle which was prepared from a capillary tube using an Automatic Magnetic Puller (Narishige Co., Tokyo, Japan). H-1/A cells were impaled with capillary needles close to the nucleus, and dye was injected by manual pressure. The transfer of dye into surrounding cells was monitored under a phase-contrast fluorescence microscope after injection. The percentage of first-order neighbors in contact with the injected cell and the number of fluorescent cells was recorded after each injection.

*Nuclear run-off transcription assay.* Run-off transcription assays were performed using a modification of the method described by Groudine et al. (12). Cells were harvested from 4 dishes by scraping with a rubber policeman. Subsequent steps were performed at 4°C. The cells were washed in phosphate-buffered saline and lysed by vortexing in NP-40 lysis buffer [10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40]. The lysates were incubated on ice for 5 minutes, resuspended in glycerol storage buffer [50 mM Tris-HCl (pH 8.0), 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA], and stored as a nucleus fraction at −80°C in 200 μl aliquots. The nucleus fraction was mixed with 200 μl of 2×reaction buffer [10 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 0.3 M KCl and 1 mM each of ATP, CTP and GTP] plus 10 μl [α-$^{32}$P]UTP (800 Ci /mmol) and incubated for 30 minutes at 30°C with shaking. After incubation, nuclei were pelleted in a microfuge, resuspended in 300 μl of 0.5 M NaCl, 50 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM Tris-HCl (pH 7.4), with 200 U of DNase I (RNase-free, Pharmacia), and incubated for 30 minutes at 37°C. After the addition of 200 μl of 5% SDS, 0.5 M Tris-HCl (pH 7.4), 0.125 M EDTA and 10 μl of 20 mg/ml proteinase K, nuclei were vortexed thoroughly and incubated for 30 minutes at 40°C. The reaction mixture was extracted three times with phenol/chloroform and the radiolabeled RNA was precipitated with an equal volume of 100% isopropanol. The precipitate was resuspended in 100 μl of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and 0.1 M NaCl, and loaded onto the Sephadex G-50 column (Boehringer Mannheim) to remove unincorporated nucleotides. An equivalent amount of radiolabeled RNA ($2\times10^6$ c.p.m) from each cell was hybridized to denatured DNA probes (>2 μg each), and immobilized to nylon filter paper in 2 ml of hybridization buffer [5×SSPE, 5× Denhardt's solution, 0.02% poly(A) and 1% SDS] for 36 hours at 65°C after preincubation in hybridization buffer for 1 hour at 65°C. The filters were washed in 2×SSC and 1% SDS twice at 65°C. The filters were exposed to X-ray film at −80°C.

## RESULTS

Marrow stroma-derived H-1/A cells had a fibroblast-like morphology and no adipocytes developed until they reached confluence. The manner of adipocyte differentiation of H-1/A cells has been described previously (23, 31, 32). In the H-1/A culture, 35.6% of the cells on the third day, 51.0% on the sixth day and 59.0% on the seventh day after confluence differentiated into adipocytes, while only 1.3% of the cells of another subline, H-1/D, did so on the seventh day after confluence.

To investigate the expression of gap-junction genes, blot hybridization of stromal cell RNA was performed with probes of connexin 43 and connexin 32 cDNA. Distinct mRNA bands of 3.0 kb were detected in all the marrow stromal cell lines previously reported (33) when tested with the connexin 43 cDNA probe, although no bands were observed in any of the cells when tested with a probe of connexin 32 (data not shown). In order to determine the level of connexin 43 transcripts in H-1/A preadipocytes and adipocytes, equal amounts of total RNA from semiconfluent and confluent cultures of H-

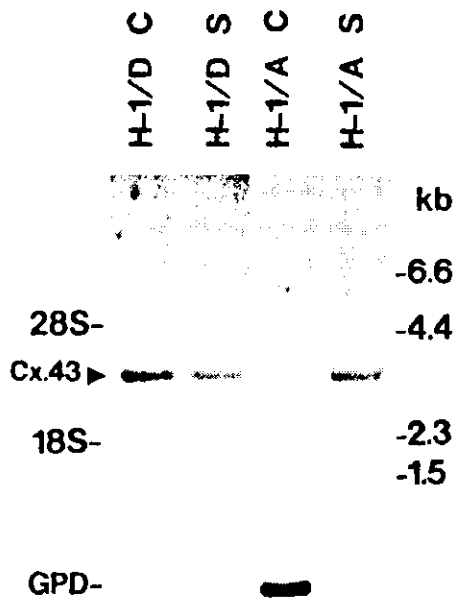


Fig. 1. Analysis of connexin 43 (Cx. 43) and GPD gene expression during adipocyte differentiation of H-1/A cells.
RNA was extracted from H-1/D cells seven days postconfluence (H-1/D C), semiconfluent H-1/D cells (H-1/D S), H-1/A cells seven days postconfluence (H-1/A C), and semiconfluent H-1/A cells (H-1/A S) (from left to right). Ten micrograms of total RNA was electrophoresed on each lane. The blot was hybridized with a probe of connexin 43 cDNA described in *Materials and Methods*. The sizes of connexin 43 and 28S and 18S rRNA markers are indicated. The same blot, which was rehybridized with the GPD probe, is shown for reference (32) in the lower panel. The autoradiograms were exposed for 72 hours at −80°C using an intensifying screen.

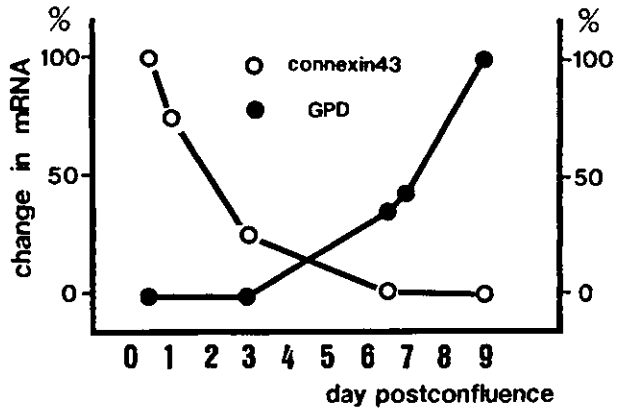


Fig. 2. Time-course of connexin 43 expression in differentiating H-1/A cells.
Connexin 43 mRNA levels (open circles) were determined densitometrically from autoradiograms of RNA blot analysis. At the indicated day of postconfluence, RNA was isolated. Five micrograms of total RNA was electrophoresed on each lane. The blots were hybridized with the connexin 43 probe. GPD mRNA levels (closed circles) are shown for reference (32). The amounts of connexin 43 mRNA at confluence (day 0) and GPD mRNA at day 9 postconfluence were regarded as equal to 100%.

1/A and H-1/D cells were subjected to blot hybridizations with the connexin 43 probe. H-1/A cells developed many fat droplets in their cytoplasm after reaching confluence, while few fat droplets were detected long after H-1/D cells reached confluence. In the confluent H-1/A culture of an adipocyte state, connexin 43 mRNA levels decreased significantly. On the other hand, no reduction of the RNA levels was observed in H-1/D cells 7 days after confluence (Fig. 1). By contrast, the levels of glycero-monophosphate dehydrogenase (GPD) mRNA clearly increased in confluent H-1/A cells as compared with those in the semiconfluent culture (32). However, no detectable bands of GPD RNA were observed in either semiconfluent or confluent H-1/D cells. Connexin 43 mRNA specifically decreased during adipocyte differentiation of H-1/A cells rather than as a result of general RNA degradation, as similar amounts of ribosomal RNA from the H-1/A cells at semiconfluence and confluence were detected on each lane. The down-regulation of connexin 43 mRNA seems to be linked with the processes of differentiation rather than with the culture condition, since another differentiation-incompetent subline, H-1/D, did not show any similar alterations in either the differentiation or the connexin 43 regulation.

To investigate the relationship between GPD and connexin 43 expression in detail, we studied the time course of their expression (Fig. 2). Immediately after confluence, connexin 43 mRNA began to decrease, whereas GPD mRNA had yet to appear. On the sixth day after confluence, connexin 43 expression continued to decrease and GPD mRNA began to be induced.

We previously reported that H-1/A preadipocytes *in*

Fig. 3. Dye transfer in H-1/A cells at semiconfluence (a, b), and at two (c, d) and four days (e, f) postconfluence.
Upper row (a, b): An H-1/A cell (asterisk) was injected with Lucifer Yellow CH at the semiconfluent stage. The fluorescent dye was extensively transferred to adjacent cells. The dye spread to three first-order neighboring cells (arrowhead, 3/3), and then to second-order neighboring cells (two arrowheads). Middle row (c, d): An H-1/A cell two days postconfluence (asterisk) was injected with Lucifer Yellow CH. The dye spread to one first-order neighboring cell (arrowhead, 1/6), but not to second-order neighboring cells. Lower row (e, f): an H-1/A cell four days postconfluence (asterisk) was injected with Lucifer Yellow CH. No dye transfer to any of the six first-order neighboring cells (arrowheads) was observed (0/6). The vacuoles in cell cytoplasm (b, d, f), which was Oil Red O-negative, were not fat droplets. Therefore, these cells containing vacuoles were not adipocytes. These cells could be easily distinguished from true differentiated cells containing fat droplets under a phase contrast microscope. Some of the cells in Fig. 4f have small fat droplets. (a, c, e) Fluorescent photographs; (b, d, f) phase contrast micrographs of (a), (c) and (e), respectively. ×400.

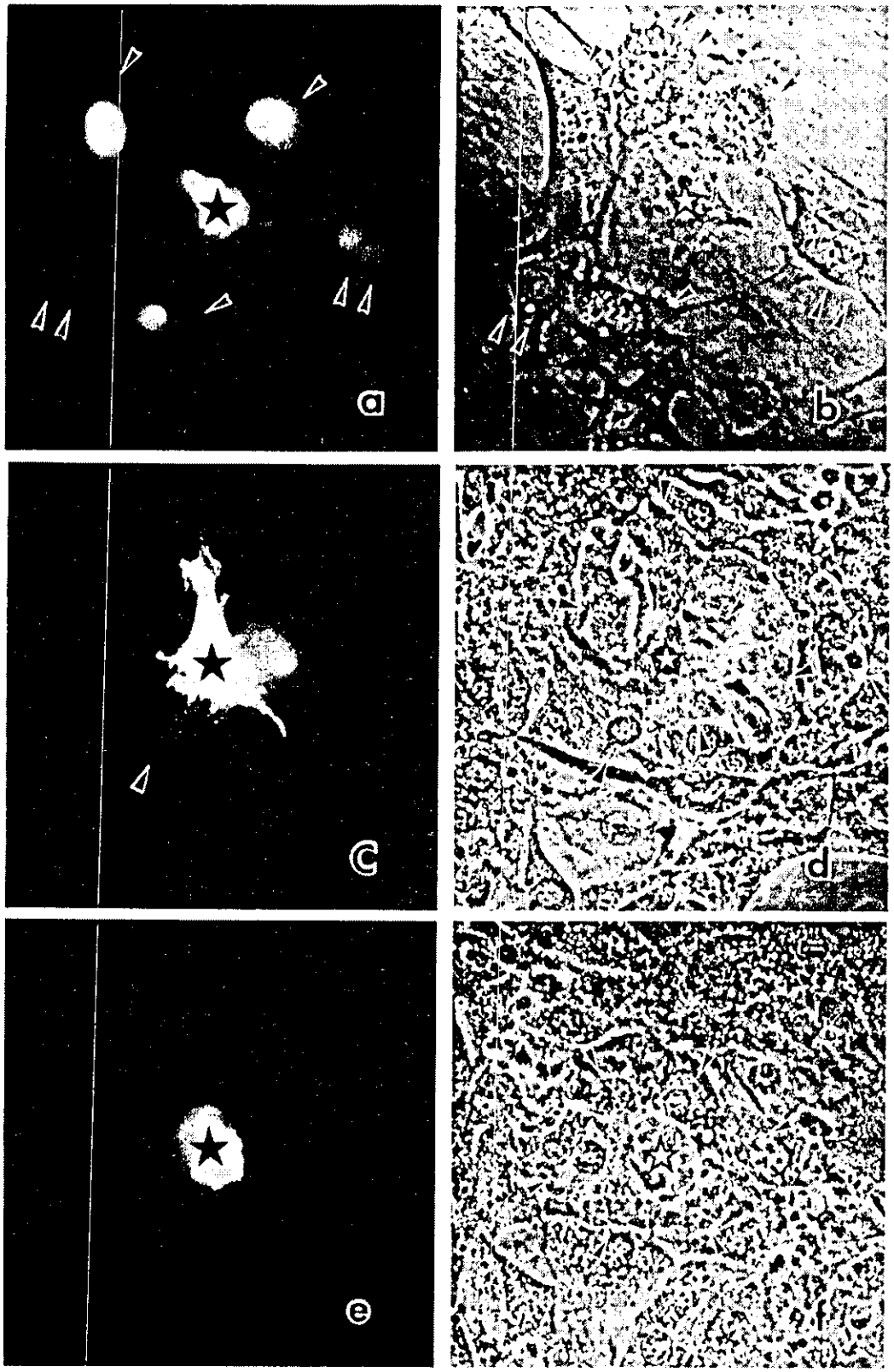


Fig. 3.

*vitro* communicate with each other via gap junctions while H-1/A adipocytes do not (30, 31). To determine whether the down-regulated expression of connexin 43 just after confluence reflects gap-junctional communication, we used the dye-transfer method to assay the communication of postconfluence H-1/A cells (Fig. 3). Gap-junctional communication decreased immediately after confluence (Fig. 4). Accompanying the decrease in communication, cells underwent a marked change in morphology during differentiation, from a flat fibroblastic form to a nearly spherical shape, as shown by the injection of Lucifer Yellow CH (Fig. 3a, c, e). The morphological change was observed before lipid accumulation in the cytoplasm.

To determine connexin 43 and GPD mRNAs during adipocyte differentiation in detail, adipocyte differentiation was inhibited by exposure to cachectin/TNF. Cachectin/TNF is widely known to inhibit adipocyte differentiation (29, 32). In order to determine whether cachectin/TNF inhibits the decrease of connexin 43 mRNA, cells were treated with 200 U/ml of cachectin /TNF when they reached confluence. At confluence, the decrease of connexin 43 expression was reduced by 200

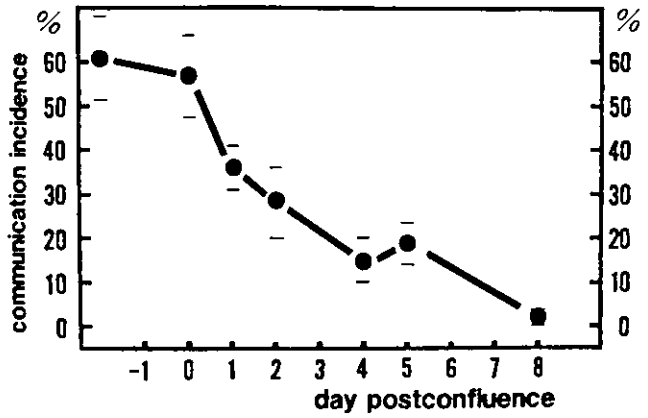


**Fig. 4.** Intercellular communication capacity of H-1/A cells postconfluence. Intercellular communication was measured by the dye transfer method. At the indicated days of postconfluent culture, Lucifer Yellow CH solution was microinjected into individual cells using a Narishige micromanipulator. The percentage of dye-transferred first-order neighboring cells was determined 10 minutes later under a fluorescence microscope. Each point represents the mean ± S.E. of 13 to 37 independent injections.

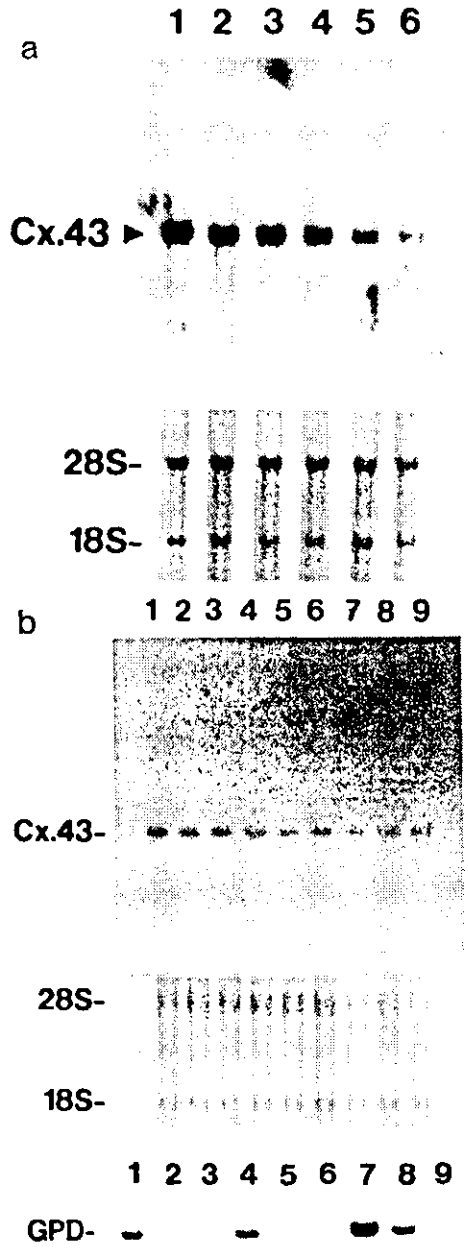


**Fig. 5.** Analysis of connexin 43 expression of H-1/A cells at confluence (a) and on day 6 postconfluence (b).
a) H-1/A cells were treated with 200 U/ml of cachectin/TNF when cells reached confluence. At confluence, no adipocytes were seen in H-1/A cells. RNA was extracted from H-1/A cells 12 hours (lane 1), 24 hours (lane 3), and 72 hours (lane 5) after treatment. Lanes 2, 4, and 6 are control cultures (without cachectin/TNF) for lanes 1, 3, and 5, respectively. b) On day 6, H-1/A cells were treated with 200 U/ml or 1,000 U/ml of cachectin/TNF. Approximately 60% of H-1/A cells differentiated into adipocytes 6 days postconfluence. RNA was extracted from H-1/A cells after a 12-hour (lane 2), 24-hour (lane 5) or 72-hour (lane 8) exposure to 200 U/ml of cachectin/TNF, or a 12-hour (lane 3), 24-hour (lane 6) or 72-hour (lane 9) exposure to 1,000 U/ml of cachectin/TNF. Lane 1 is a control (without cachectin/TNF) for lanes 2 and 3; lane 4, for lanes 5 and 6; and lane 7, for lanes 8 and 9. The blot was hybridized with the connexin 43 probe first. Then, the blot was dehybridized with boiled 0.01% SDS, 0.01 × SSC solution 5 times, and rehybridized with the GPD probe (b, bottom). The conditions of hybridization are described in *Materials and Methods*. Methylene blue-stained 28S and 18S rRNA from the gel is shown (a, lower part; b, middle part).

U/ml of cachectin/TNF (Fig. 5a). GPD, a key enzyme of fat synthesis, was not expressed in either the control or cachectin/TNF-treated cultures. Almost no H-1/A cells differentiated into adipocytes with the addition of cachectin/TNF 12, 24, and 72 hours after confluence. Approximately 20% of the untreated control cells differentiated into adipocytes 72 hours after confluence while no untreated cells differentiated 12 and 24 hours after confluence. In order to determine whether cachectin /TNF reverses the decrease of connexin 43 transcript in the differentiated H-1/A cells, 6 days after confluence, cells were also treated with 200 U/ml or 1,000 U/ml of cachectin/TNF. On the sixth day after reaching confluence, H-1/A expressed both connexin 43 and GPD (Fig. 6b). Almost 60% of the untreated H-1/A cells differentiated into adipocytes six days after reaching confluence. In the control culture, connexin 43 expression decreased while the GPD expression increased (Fig. 5b, lanes 1, 4, 7). With 1,000 U/ml of cachectin/TNF, the decrease of connexin 43 mRNA was again reduced. The amounts of these mRNAs increased or decreased specifically, rather than as a result of general RNA degradation, for almost the same amounts of ribosomal RNAs of H-1/A cells were detected in the control and cachectin/TNF-treated cultures (Figs. 5a and 5b). In both the undifferentiated and differentiated H-1/A cells, cachectin/TNF reduced the decrease of connexin 43 transcript.

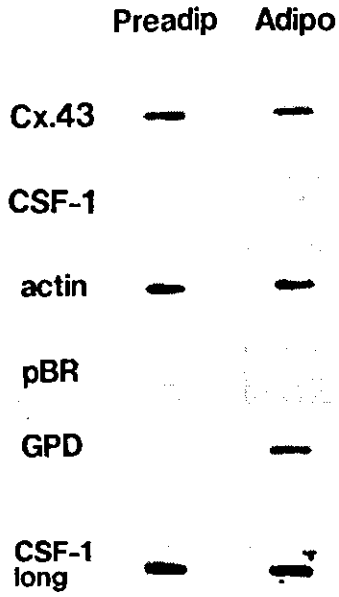


**Fig. 6.** Nuclear run-off transcription assay of H-1/A at semiconfluence and postconfluence.
Semiconfluent H-1/A (Preadip) and H-1/A cells at 7 days postconfluence (adipo) were harvested from two dishes, and nuclei were isolated. An equivalent amount of radiolabeled RNA ($2 \times 10^6$ c.p.m) from each cell was hybridized to denatured indicated DNA probes ($>2\,\mu g$ each) immobilized to nylon filter paper in 2 ml of hybridization buffer (5×SSPE, 5×Denhardt's solution, 0.02% poly(A) and 1% SDS) for 36 hours at 65°C after preincubation in hybridization buffer for 1 hour at 65°C. The filters were washed in 2×SSC and 1% SDS twice at 65°C. The autoradiogram was exposed for 48 hours (connexin 43, CSF-1, actin and pBR) or seven days (GPD and CSF-1 long) at −80°C, using an intensifying screen.

To determine whether the decrease in connexin 43 cytoplasmic RNA was due to a decreased rate of transcription or certain posttranscriptional events, we also performed nuclear run-off transcription assays. In these experiments, nuclear RNA was radiolabeled by *in vitro* RNA elongation while reinitiation of RNA synthesis was prevented. The transcriptional rate of connexin 43 genes during differentiation was estimated by measuring the amount of labeled RNA transcribed in nuclei isolated from the H-1/A cells. To demonstrate the specificity of the observed signals, the labeled nuclear RNA was hybridized to pBR 322. No detectable signals were obtained. The amount of connexin 43-specific transcription was quantitated by hybridization to the excess connexin 43 cDNA bound to a nylon filter. When cells differentiated into adipocytes, the transcriptional rate of connexin 43-specific RNA decreased to 65% in densitometric analysis, while the transcriptional rate of CSF-1-specific RNA did not change significantly, as described previously (Fig. 6, ref. 32). The transcriptional rate of the actin gene is decreased as well after adipocyte differentiation, as previously reported (32). From these results, we conclude that the decrease of connexin 43-specific cytoplasmic RNA is due, in part to, the change in the transcriptional level. We also attempted to determine the half-life of connexin 43 mRNA in adipocytes, but were unable to do so, since almost no connexin 43 expression was observed in adipocyte RNA even when cells were not exposed to actinomycin D.

## DISCUSSION

With the electron microscope, gap junctions are observed between marrow stromal cells *in vivo* (36). In adherent cells of long-term bone marrow culture and stromal cell lines, *in vitro* communication between cells via gap junctions has been observed by electron microscopy and with the dye-transfer method (30, 38). In this study, marrow stromal cells were found to express connexin 43 but not connexin 32. Connexin 43 is a heart-muscle-type connexin , and is expressed in heart, ovary, kidney, uterus and lens (4). In contrast, connexin 32 is expressed in liver, kidney, brain, and stomach (24). The type of connexin is the same in marrow stroma irrespective of differentiation capability, i.e., in adipocytes, osteocytes and myotubes. The myotube differentiation of KUSA cells is consistent with the fact that KUSA cells expressed the heart-muscle-type connexin (33).

Gap-junctional communication is regulated by vari-

ous cytoplasmic factors such as protein kinase C, cAMP, $Ca^{++}$ ion, and pH (8, see also reviews, ref. 21). These factors change the communication level by modulating the gap-junctional structure directly or indirectly. The phosphorylated state of gap-junctional protein is related to junctional communication (22). Crystallization of the gap-junctional component is associated with junctional uncoupling in an ultrastructural study (14). The radial displacement of connexin at the cytoplasmic end is thought to result in closing of the gap-junctional channel (34). In the H-1/A cells used in this study, gap-junctional communication was reduced after adipocyte differentiation. Our time-kinetic study of connexin 43 expression clearly demonstrated that the loss of gap-junctional communication after the adipocyte differentiation of H-1/A cells is explained by changes in the connexin 43 mRNA levels, not by those in the the protein level or phosphorylated state.

The down-regulation of connexin 43 expression began prior to the switching-on of the lipogenic enzyme gene, and is, therefore, one of the early events in the differentiation process. Just after confluence, cell growth is arrested (23) and cell shape is changed in H-1/A cells. Therefore, down-regulated expression of connexin 43 was accompanied by changes in cell morphology and cell growth at the early stage of differentiation, independent of lipid accumulation. These changes in connexin 43 during differentiation are similar to those in cytoskeletal proteins such as actin and tubulin (28, 32). The change in cell morphology during adipocyte differentiation is independent of lipid accumulation (20) and is accompanied by alterations in cytoskeletal proteins (28).

Various levels of gene control have been reported in eukaryotic cells during differentiation (7). The quantitative data obtained by nuclear run-off transcriptional assays clearly showed that the decreased transcriptional rate of connexin 43 gene during adipocyte differentiation is similar to that of cytoskeleton (actin) genes (32). In contrast, the reduction of transcripts of CSF-1, a hematopoietic growth factor, after adipocyte differentiation is due to a posttranscriptional event rather than a decreased rate of transcription; connexin 43 and CSF-1 mRNAs are down-regulated by different mechanisms during the differentiation of H-1/A cells.

*In vivo*, marrow adipocytes, which have a basement membrane around the cells, do not have gap junctions with neighboring cells, while most fibroblasts do (36). These ultrastructural findings are consistent with results in studies indicating that stromal cells lose their gap-junctional communication after adipocyte differentiation.

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# Colony-Stimulating Factor 1 Expression Is Down-Regulated during the Adipocyte Differentiation of H-1/A Marrow Stromal Cells and Induced by Cachectin/Tumor Necrosis Factor

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**We isolated clonal sublines of the established mouse marrow stromal cell line, H-1. These clonal sublines underwent differentiation into adipocytes in various degrees. One subline, H-1/A, underwent adipocyte differentiation after confluence, while another subline, H-1/D, did not differentiate. In H-1/A cells, the 4.5- and 2.5-kb major mRNA species of colony-stimulating factor 1 (CSF-1) were expressed before differentiation and were down-regulated at a posttranscriptional level during the differentiation of H-1/A cells. The down-regulation of the CSF-1 gene was not a result of arrested cellular growth, because no down-regulation was detected in the nondifferentiating sister line, H-1/D. This down-regulation appeared to be an early event in differentiation. Cachectin/tumor necrosis factor transiently induced the expression of CSF-1 and inhibited the differentiation of H-1/A cells into adipocytes. This induced expression of CSF-1 was due to an increased rate of transcription.**

Colony-stimulating factors (CSFs) are a family of glycoproteins required for the survival, proliferation, and differentiation of the hematopoietic cells. Murine CSFs have been molecularly cloned and characterized on the basis of their specificity for the direction of hematopoietic differentiation (9, 12, 25, 34, 48). CSF-1, one of the CSFs, is secreted by certain organs or cells (17, 18, 34–37, 42) and by marrow stromal cell lines in vitro (10), and it stimulates the differentiation of certain hematopoietic stem cells into a monocytic lineage (14, 45, 46). The CSF-1 receptor belongs to a single class of high-affinity receptors and appears to be identical to the product of the c-*fms* proto-oncogene (41), which exhibits tyrosine kinase activity (28), as does the *src* gene product (20). Cotransfection of NIH 3T3 cells with CSF-1 and the c-*fms* gene induces cell transformation by an autocrine mechanism (38–40).

On the other hand, cachectin/tumor necrosis factor (TNF), one of the cytokines, is reported to induce the expression of granulocyte-macrophage CSF (GM-CSF) and granulocyte CSF (G-CSF) in lung fibroblasts (22, 29) and endothelial cells (3) and to repress the expression of lipogenic enzymes (47). The effects of cachectin/TNF seem to reflect one of the physiological bases for cachexia in patients with certain infections and malignancies (31, 43).

A marrow stromal cell line, H-1/A, cloned from the bone marrow of C57/B1 mice, produces CSFs and differentiates into adipocytes (15). We previously reported that colony-stimulating activity of H-1/A cells stimulated the growth and differentiation of granulocytes (30). Colony-stimulating activity of H-1/A cells decreases when the cells differentiate into adipocytes. Thus, this marrow stromal cell line seems to play important roles in the regulation of hematopoiesis by the secretion of CSFs and by their down-regulation in adipocyte differentiation. However, the regulatory mechanism of CSF production remains obscure in the marrow stromal cell. This study reports that the down-regulation of CSFs was controlled at a posttranscriptional level and preceded the switch-on of the glyceromonophosphate dehydrogenase (GPD) gene during the adipocyte differentiation of H-1/A cells. We also demonstrate that cachectin/TNF inhibited both adipocyte differentiation and the switch-on of a lipogenic enzyme gene and induced the expression of CSF-1 at a transcriptional level in the H-1/A cells.

## MATERIALS AND METHODS

**Cell culture.** Sublines were cloned from H-1 cells by dilution plating and then routinely cultured with Fischer medium (GIBCO, Grand Island, N.Y.) supplemented with 10% horse serum (Irvine Scientific, Santa Ana, Calif.), penicillin, and streptomycin. The cultures were incubated at 33°C in an atmosphere of 5% carbon dioxide. In certain cases, the growth medium was supplemented with $10^{-6}$ M hydrocortisone (Upjohn Co., Kalamazoo, Mich.) or with 1 mM dibutyryl cyclic AMP (dbcAMP) (Sigma, St. Louis, Mo.) and 1 mM of caffeine. H-1/A cells were also treated with 200 U of cachectin/TNF (Genzyme Co., Boston, Mass.) per ml at confluence. To estimate adipocyte differentiation, dishes were fixed with 10% buffered neutral Formalin and stained with 0.5% oil red O in propylene glycol. Four photomicrographs were randomly taken for each dish, and obvious fat droplets in the cytoplasm were designated as markers of differentiation (13, 16, 49, 50). About 2,000 cells were then counted in each dish, and the percentage of adipocytes was calculated.

**Isolation of adipocytes.** Adipocytes were isolated by the procedure of Spiegelman et al. (44). Cells were harvested by treatment with trypsin and were centrifuged twice at 2,500 × *g* for 5 min to pellet the preadipocytes in 7 ml of phosphate-buffered saline (PBS) containing 1 mM of phenylmethylsulfonyl fluoride. The supernatant which contained adipocytes was gently mixed with monobromobenzene-saturated PBS and was recentrifuged in PBS saturated with bromobenzene.

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In order to harvest the cells from the dishes, the cells were scraped off with a rubber policeman.

**DNA probes.** The CSF-1 probe was a 3.9-kb *Eco*RI fragment from pGEM2MCSF-10 (a gift from J. S. Price, Cetus Co.) (25). pGEM2MCSF-10 contained 3.9-kb CSF-1 cDNA cloned from mouse L cells. The G-CSF probe was a 1-kb *Bam*HI-*Eco*RI fragment from pMG-3 ( a gift from S. Nagata) (48). The interleukin-3 (IL-3) probe was a 0.6-kb *Pst*I-*Nco*I fragment from pILM-3 (a gift from I. G. Young) (9). The GM-CSF probe was a 0.75-kb *Bam*HI-*Eco*RI fragment from pGMG3.2 (a gift from A. R. Dunn) (12). The GPD probe was a 0.9-kb *Eco*RI fragment of pL10.9 (a gift from B. M. Spiegelman) (44). The actin probe was a 6.8-kb *Eco*RI fragment of pSP62-PL obtained from N. Davidson (19).

**RNA blot analysis.** Fresh growth medium or medium containing 200 U of cachectin/TNF per ml was added to semiconfluent or confluent layers of H-1/A cells. After incubation, cells were harvested for RNA blot analysis. In another experiment, fresh growth medium or medium containing $10^{-5}$ M l-epinephrine, 10 μg of phytohemagglutinin per ml, 5 μg of concanavalin A (Sigma) per ml, $10^{-6}$ M hydrocortisone, 0.5 mM dbcAMP, and 1 mM caffeine with 10 or 100 U of recombinant IL-1α, 1 or 10 U of recombinant IL-1β (Cistron Bio., Pine Brook, N.J.) per ml, or 10 μg, 1 μg, 100 ng, 10 ng, or 1 ng of lipopolysaccharide (Sigma) per ml was added to the semiconfluent layers of H-1/A cells. After incubation for 12 h, cells were harvested. Purified recombinant IL-1α (26) was a gift from P. Lomedico (Hoffman-La Roche, Nutley, N.J.).

RNA was prepared from cultured cells by homogenization in guanidinium isothiocyanate, followed by centrifugation over a cesium chloride cushion (4). The RNA was then electrophoresed in a 1.0% agarose gel, transferred to a Nylon filter (Du Pont Company NEN Products), and hybridized with cDNA inserts labeled with [$^{32}$P]CMP by random-primer method (8) at 65°C for 14 to 16 h in buffer containing 5× SSPE (1× SSPE is 0.75 M NaCl, 0.05 M NaCl, 0.004 M EDTA), 5× Denhardt solution, 0.02% poly(A), and 1% sodium dodecyl sulfate (SDS). The blots were washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 1% SDS at room temperature and 65°C. Final washings were done with 0.1× SSC containing 0.1% SDS at 65°C. The blots were exposed to X-ray film at −80°C, using an intensifying screen. The amounts of RNA hybridized to each probe were determined by densitometry of bands, using Quick Scan R & D (Helena Laboratories).

**Bone marrow colony-forming units in culture (CFUc) assay.** The medium was harvested from cachectin/TNF-treated or untreated H-1/A cells grown for 3 days in Fischer medium supplemented with 10% fetal bovine serum and antibiotics. It was filtered through a Millipore filter (pore size, 0.22 μm) and stored at −20°C until use. CFUc were allowed to proliferate in semisolid soft agar cultures as previously described (33). Fresh murine bone marrow cells were obtained from 8- to 12-week-old female C57/BL mice by flushing their tibias and femurs. Fresh marrow cells were inoculated in 1 ml of complete Iscove medium containing 0.3% agar (Difco Laboratories, Detroit, Mich.) and 10% appropriate conditioned medium in plastic petri dishes. After incubation for 6 days at 37°C in an atmosphere of 7.5% $CO_2$ in humidified air, the specimens were air-dried and stained by means of a double-staining technique using naphthol-ASD-chloroacetate and α-naphthyl butyrate as substrates (27).

**Nuclear run-off transcription assay.** Run-off transcription assays were performed by using a modification of the method described by M. Groudine et al. (11). Nuclear RNA was radiolabeled by in vitro RNA elongation while reinitiation of RNA synthesis was prevented. Cells were harvested from four dishes by scraping with rubber policemen. Subsequent steps were performed at 4°C. The cells were washed in PBS and lysed by vortexing in Nonidet P-40 lysis buffer (10 mM Tris hydrochloride [pH 7.4], 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40). The lysates were incubated on ice for 5 min, resuspended in glycerol storage buffer (50 mM Tris hydrochloride [pH 8.0], 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA), and stored as a nucleus fraction at −80°C in 200-μl aliquots. The nucleus fraction was mixed with 200 μl of 2× reaction buffer (10 mM Tris hydrochloride [pH 8.0], 5 mM $MgCl_2$, 0.3 M KCl, 1 mM each ATP, CTP, and GTP, and 10 μl of [α-$^{32}$P]UTP [800 Ci/mmol]) and incubated for 30 min at 30°C with shaking. After incubation, nuclei were pelleted in a microfuge, resuspended in 300 μl of a solution consisting of 0.5 M NaCl, 50 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM Tris hydrochloride (pH 7.4) with 200 U of DNase I (RNase-free, Pharmacia), and incubated for 30 min at 37°C. After the addition of 200 μl of a solution consisting of 5% SDS, 0.5 M Tris hydrochloride (pH 7.4), 0.125 M EDTA, and 10 μl of 20-mg/ml proteinase K, nuclei were vortexed thoroughly and incubated for 30 min at 40°C. The reaction mixture was extracted three times with phenol-chloroform, and the radiolabeled RNA was precipitated with an equal volume of 100% isopropanol. The precipitates were resuspended in 100 μl of a solution consisting of 10 mM Tris hydrochloride (pH 8.0), 1 mM EDTA, and 0.1 M NaCl and loaded onto the Sephadex G-50 column (Boehringer Mannheim) to remove unincorporated nucleotides. An equivalent amount of radiolabeled RNA ($2 \times 10^6$ cpm) from each culture was hybridized to denatured DNA probes (>2 μg each), immobilized to Nylon filter paper in 2 ml of hybridization buffer [5× SSPE, 5× Denhardt solution, 0.02% poly(A), 1% SDS] for 36 h at 65°C after preincubation in hybridization buffer for 1 h at 65°C. The filters were washed in 2× SSC–1% SDS twice at 65°C. The filters were exposed to X-ray film at −80°C. The amounts of RNA hybridized to each probe were determined. The densitometry was done in the same way as in the blot analysis.

## RESULTS

All the sublines of H-1 cells had a fibroblastlike morphology, and no adipocytes developed until they reached confluence. Sublines showed various degrees of differentiation into adipocytes at confluence. The manner of adipocyte differentiation of a subline H-1/A was described previously (30). In the H-1/A culture, 59% of the cells differentiated into adipocytes on the seventh day after confluence, while only 1% of the cells did so in subline H-1/D. It has been reported that hydrocortisone or dbcAMP and caffeine modulate adipocyte differentiation (16, 30). However, we found no effect on H-1/A cells (data not shown).

Blot hybridization of H-1/A RNA isolated from the semiconfluent culture was done with probes of mouse CSF-1, GM-CSF, G-CSF, and IL-3 cDNA (9, 12, 25, 48). Distinct 4.5- and 2.5-kb mRNA bands were detected in the semiconfluent H-1/A cells with the CSF-1 cDNA probe (Fig. 1a), while no bands were observed with the probes of GM-CSF, G-CSF, and IL-3 cDNA (data not shown). To characterize the difference between the 4.5- and 2.5-kb CSF-1 transcripts, the blots of H-1/A RNA were hybridized with different fragments of the CSF-1 cDNA. The two species of CSF-1 transcripts differed in their 3′ sequences (data not shown).