Contact- and Growth Factor-Dependent Survival in a Canine Marrow-Derived Stromal Cell Line

By Ralf Huss, Cynthia A. Hoy, and H. Joachim Deeg

Cell-cell interactions and the presence of growth factors such as stem cell factor (SCF; or c-kit ligand) or interleukin-6 (IL-6) are involved in the proliferation and differentiation of the canine marrow-derived stromal cell line DO64. In the presence of SCF, stromal cells are induced to differentiate, but not to proliferate. In contrast, in the presence of IL-6, stromal cells are induced to proliferate rather than to differentiate in culture. Both SCF and IL-6 are produced by the stromal cells themselves and, thus, act as autocrine factors. In addition, DO64 cells also interact physically with each other in culture when grown under optimal culture conditions (70% to 90% cell confluence) and in the presence of serum, thereby supporting proliferation and maintaining viability. Under conditions of lower cell density or low serum or growth factor concentrations in culture, DO64 cells tend to aggregate and form clusters. This increase in local cell concentration is associated with preservation of viability, presumably because of the accumulation of autocrine factors. If no signal, neither intercellular nor soluble, is provided, and DO64 cells are not able to reach a critical cell density or to produce sufficient factors in an autocrine fashion, the cells cease to proliferate and eventually die.

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From the Transplantation Biology Program, Division of Clinical Research, Fred Hutchinson Cancer Research Center, Seattle, WA. Submitted October 21, 1994; accepted December 15, 1994.

Supported in part by Grants No. CA18221, CA31787, DK42716, CA 18029, and HL36444 from the National Institutes of Health, Department of Health and Human Services, Bethesda, MD. R.H. was a recipient of a grant of the Deutsche Forschungsgemeinschaft, Bad Godesberg, Germany. H.J.D. is also supported by a grant from Baxter Health Care Division (Deerfield, IL)/National Marrow Donor Program.

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0006-4971/95/8509-06$3.00/0

tion of 4 μg/mL and added to cell cultures. The cell cultures were then examined under an immunofluorescence phase-contrast microscope (Zeiss, Axiovert, Germany) and the proportion of red (PI) stained dead cells was estimated. In addition, stromal cells were stained with a 1:1 mixture of acridine orange (AO) and ethidium-bromide (EB). 100 μg/mL of each stain were mixed in phosphate-buffered saline (PBS). From adherent growing stromal cells, culture supernatant was discarded from the tissue-culture flask and 10 μL of dye mix was added. The flask was gently shaken by hand and then examined with a fluorescence microscope (Zeiss) using epilumination and a filter combination for observing fluorescence.

**Growth factors.** Where available, we used canine growth factors for our assays. All other growth factors were cloned from human cDNA. Canine stem cell factor (SCF; c-kit ligand) was provided by Amgen (Thousand Oaks, CA). Dr R. Nash (Fred Hutchinson Cancer Research Center, Seattle, WA) provided canine GM-CSF, and Dr D. Gebhard (North Carolina State University, Raleigh, NC) provided the IL-6-producing canine cell line 030-E. Immunex Corp (Seattle, WA) provided the following human growth factors: interleukin-1α (IL-1α), IL-2, IL-3, IL-7, IL-15 and leukemia inhibitory factor. Human IL-l and human insulin-like growth factor 1 were purchased from Genzyme (Cambridge, MA).

**Assays for CFUs.** Nonadherent DO64 cells (see above) were removed from culture flasks and placed at a concentration of 5 × 10^4 cells/dish in semisolid agar for a standard CFU assay as described by Dexter et al. and modified for canine cells by Schuening et al.

Similarly, adherent cells were detached by brief trypsin treatment, washed in medium, and placed in semisolid agar at 5 × 10^4 cells/dish. Fresh mononuclear marrow cells served as controls.

**Proliferation assay.** Stromal cells were grown in 96-well plates (Costar, Cambridge, MA) for 24 hours at a cell density of ≈70%. Cells were then incubated with various growth factors for 24 hours. The optimal concentration for each factor was determined in ancillary experiments. Cells in culture medium without added factors served as controls. After 24 hours, each well was pulsed with 1 μCi ³H-thymidine (Amersham, Arlington Heights, IL) and cells were harvested 6 hours later according to standard procedures. The trapped ³H-activity was measured in a beta-scintillation counter (LS6000SC, Beckman Instruments, Irvine, CA). Cultures were assayed in triplicate, and the mean was calculated.

**Immunphenotyping.** DO64 cells were grown in 24-well tissue-culture plates (Costar, Cambridge, MA) in the presence of SCF (100 ng/mL), IL-6 (50% cell line supernatant) or serum-free medium.

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**Fig 1.** Transcription of growth factors in DO64 cells. RNA was isolated from DO64 cells and hybridized with canine or human probes for various growth factors as described elsewhere. The pattern of growth factor transcription is representative of a marrow stromal cell line.

**Fig 2.** DO64 stromal cells were grown in 24-well plates under different conditions. At various time points, the tissue-culture medium was discarded and the cells were stained with a 1:1 dye mixture of AO and EB. After 20 minutes, cells were examined under a fluorescence microscope (Zeiss Axiovert, Jena, Germany) (original magnification × 10). (A) Stromal cells grown at high density (70% to 90%) and in serum-containing medium (10% to 20%) under optimal growth conditions. All cells grow adherent and are viable. (B) Stromal cells in culture grown at low-density (30% to 50%) but in serum-containing medium. Aggregation of stromal cells ("clusters") is associated with increased viability of stromal cells and a decrease in the number of cells, which undergo apoptosis (24 hours). (C) The withdrawal of serum from the medium increases the number of nonadherent cells and the proportion of cells which undergo apoptosis (48 hours). (D) All single cells are dead, whereas cells in clusters remain viable (72 hours).
Table 1. Growth Characteristics of D064 Cells at Different Cell and Serum Concentrations

<table>
<thead>
<tr>
<th>Cell Confluence</th>
<th>Serum Concentration</th>
<th>Comment</th>
<th>Figure</th>
</tr>
</thead>
<tbody>
<tr>
<td>70%-90%</td>
<td>10%-20%</td>
<td>Optimal growth conditions, no cluster formation</td>
<td>2A</td>
</tr>
<tr>
<td>30%-50%</td>
<td>5%</td>
<td>Beginning cluster formation, still high viability (24 hrs)</td>
<td>2B</td>
</tr>
<tr>
<td>30%-50%</td>
<td></td>
<td>Increasing cell death, viable clusters (48 hrs)</td>
<td>2C</td>
</tr>
<tr>
<td>30%-50%</td>
<td></td>
<td>After 72 hours, only large clusters are viable</td>
<td>2D</td>
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</table>

The cells were observed over 72 hours after a serum-depletion (Fig 1).

After 48 hours, the wells were carefully washed with PBS and fixed with ethanol for 10 minutes. After drying overnight, the wells were stained with the primary antibody for 30 minutes. The irrelevant antibody G3G6 (C. Badger, personal communication) recognizing gpIIb/IIIa on human platelets was used as a control. After washing the wells again with PBS, the cells were incubated with a secondary FITC-conjugated goat-antimouse antibody and analyzed by fluorescence microscopy (Zeiss).

**Time-lapse video recording.** Stromal cells, plated at a concentration of 5 × 10⁶ cells/mL, were grown in 24-well tissue-culture plates (Costar, Cambridge, MA) in the presence of soluble SCF (100 ng/mL). The tissue-culture plate was placed under a phase-contrast microscope (Zeiss) and photographs were taken from identical fields every 15 minutes for 48 hours (original magnification × 16) by a CCD video camera (Applied Precision, Mercer Island, WA). The picture sequences were analyzed by a computer modeling system (ISEE, Inovision Corp, Research Triangle Park, NC) and transferred to a computer high-speed summary of the recording period.

**Electron microscopy.** Transmission electron microscopy (TEM) was performed as described. Briefly, stromal cells, grown in chambers on circular cover slips (NUNC, Naperville, IL), were fixed in Karnofsky's/Karnofsky 2 and medium 1:1, washed in 2% OsO₄ for 1 hour, and dehydrated in ethanol at increasing concentrations (35%, 50%, 95%, and 100%, each for 15 minutes). Preparations were then incubated again with Karnofsky's/Karnofsky 2 and medium (1:1) and placed in 100% EPON 812 (Polyscience, Warrington, PA) for 1 hour. This was followed by 100% EPON 812 overnight and embedded in fresh EPON 812 by inverting BECM capsules filled with EPON 812 on appropriate areas and cured for 48 hours in a 60°C oven. Preparations were sectioned on a Porter-Blum MT-1 ultramicrotome with a Microtome diamond knife (Newton, MA).

For examination, Tsousinius 200 mesh grids (Rockville, MD) were used, double stained with saturated 6% uranyl acetate for 2 hours, rinsed, exposed to Millonig's lead stain for 4 minutes, rinsed again, and examined on a JEOL 100 SX electron microscopy (JEOL Inc., Peabody, MA).

**Cell-cycle analysis.** For cell-cycle analysis, D064 cells were either grown in serum-containing medium (10% FCS) or were incubated with SCF (100 ng/mL) or IL-6 containing supernatant (1 mL/4 mL tissue-culture medium) from the cell line D064-E. After 24 hours, cultures were treated with trypsin (0.05%) for 15 minutes at 37°C, and 1 × 10⁶ cells were washed twice in 1% HBSS. The cellular pellet was resuspended in 250 μL of buffer A (10 mmol/L TRIS-HCl, pH 7.5, 20 mmol/L NaCl, 20 mmol/L MgCl₂) and kept on ice for 5 minutes. Buffer B was added (50% buffer A, 1% NP40) and the mixture was incubated for 5 minutes on wet ice. Then 10 μL of DNase-free RNase (final 10 μg/mL; Boehringer Mannheim) was added and left at 37°C for 30 minutes. Finally, 500 μL of PI solution (100 μg/mL PI, Sigma; 0.1% Triton x100, USB; fetal bovine serum; HyClone, Logan, UT) was added to each tube and left in the dark at 4°C for 1 hour before reading. The DNA content was determined by FACScan analysis using LYSIS II software (Becton Dickinson) and cell-cycle analyses were performed with the Multicycle computer program after analyzing the data by Reproman software (Finn Fads Software, Seattle, WA).

**RESULTS**

**Optimal growth conditions for stromal cells in culture.** The growth of D064 cells was verified by daily inspection and estimation of the number of cells in culture. Optimal growth conditions were assessed by the ability of D064 cells to maintain viability and to divide. The doubling time of D064 cells was approximately 48 hours. Factors determined to be necessary for optimal growth conditions were cell density in culture and the presence of a sufficient concentration of serum or conditioned medium. The cell density necessary to maintain optimal growth and viability of stromal cells in culture was ~70% to 90% confluency. If serum (eg, FCS) was added for a final concentration of 10% to 20%, proliferation and growth of D064 cells were further enhanced. The presence of conditioned medium (CM) derived from the cell-free supernatant of stromal cells grown to high density, allowed D064 cells at a lower confluence in culture to grow while maintaining proliferation and viability. Stromal cells grown at a density of 70% to 90% in serum-free medium, or at a lower confluence (50% to 70%), but in the presence of 10% to 20% serum or 30% to 50% CM, showed optimal viability and proliferation rates, suggesting that a factor (factors) present in serum or CM could functionally substitute for signals otherwise provided by high cell density or cell-cell contact.

**Cluster formation prolongs cell survival.** The analysis of cell growth and survival of canine marrow-derived cells in culture suggested that stromal cells did not necessarily require signals from other cell types to survive: optimal growth conditions were provided by increasing cell density in culture. Necessary signals were apparently provided by stromal cells in an autocrine fashion. A certain cell density was required to facilitate close contacts among stromal cells and possibly to produce autocrine factors at sufficient concentrations. As shown in Fig 2A, stromal cells grown in the presence of 10% FCS and at a density of 70% maintained a viability of 99% and complete adherent growth. When D064 cells were grown at only 50% confluence and the serum-concentration was reduced to 5%, cells started to form clusters (within 24 hours), part of which continued to grow (Fig 2B). However, D064 cells contained in clusters still remained viable. At 72 hours, only the largest clusters (>30 to 40 cells) remained viable (Fig 2D). These findings suggested that stromal cells were able to maintain viability under suboptimal conditions by increasing their local or regional density by forming cell clusters. The cluster formation, which concentrated a large number of cells in a small volume, presumably provided the
Canine marrow-derived stromal cells were grown in serum-containing medium in 24-well tissue-culture plates. The plates were placed under a time-lapse camera for 48 hours and photographed every 15 minutes. This sequence illustrates cell-cell interactions of DO64 cells (arrows), including cell detachment and later reattachment after contact with an adherent cell. The amount of extracellular matrix (ECM) increases with time in culture (original magnification × 16).

**Fig 3.**

**Cell-cell interaction.** Time-lapse analysis of stromal cells under optimal conditions in culture over 48 hours showed direct cell-cell contacts among DO64 cells (Fig 3). Over the time period studied, the stromal cells came in physical contact with each other. Some cells started to round up and then detached, but remained viable. These nonadherent cells remained in culture as floating cells until they came again in physical contact with other adherent cells, reattached and resumed growth in an adherent fashion. We have shown previously that DO64 cells that detach are able to differentiate.
Fig 4. Colony formation of normal marrow and D064 cells in semisolid agar. In each dish, $5 \times 10^4$ cells were plated and colonies (CFU-GM) were counted at 14 days. All assays were done in triplicate. Shown are results of one experiment (triplicate cultures) with normal marrow mononuclear cells, spontaneously detached (nonadherent) D064 cells, and adherent D064 cells detached by trypsin treatment. Results with trypsin-treated detached D064 cells (not shown) were not different from those with untreated cells. Adherent cells failed to form distinct colonies, but continued to produce a layer of fibroblast-like cells.

...and exhibit hematopoietic features. The detachment is further enhanced by the addition of SCF (see below). In contrast, adherent cells fail to form hematopoietic colonies in semisolid agar and continue to form a layer of fibroblast-like stromal cells (Fig 4). These observations suggest that D064 cells not only interact physically with each other under stress conditions (cluster formation during factor withdrawal), but also receive signals, presumably via membrane receptors that play a role in the decision of these cells to differentiate.

Factors involved in stromal cell differentiation and proliferation. We then examined the effect of two soluble factors which are thought to be involved in the differentiation and proliferation of stromal cells. D064 cells, which detached, began to express differentiation markers such as CD34 and MHC class II (DR) and showed the morphology of differentiating progenitors. The addition of soluble SCF (10-500 ng/mL) as the sole exogenous growth factor resulted in an increase in the number of nonadherent DR$^+$ cells in culture. SCF did not enhance proliferation of stromal cells. In contrast, interleukin-6 (IL-6) or conditioned medium from D064 cells were potent stimulators of proliferation (Fig 5), but failed to induce differentiation of stromal cells. None of the other factors tested induced either differentiation or proliferation. Adherent growing D064 cells also increased significantly in number when IL-6 was present in culture. However, in contrast with observations in cultures containing serum, exogenous SCF or cells at high density, hardly any nonadherent cells emerged (Fig 6). The response of D064 cells to a combination of SCF and IL-6 was dependent on the concentration of each factor. Concentrations of IL-6 (added as su-
Table 2. Effect of Culture Conditions on Cell Cycle Distribution

<table>
<thead>
<tr>
<th>Culture Condition</th>
<th>G1 Phase (%)</th>
<th>S Phase (%)</th>
<th>G2 Phase (%)</th>
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<tbody>
<tr>
<td>Medium</td>
<td>71 ± 4</td>
<td>10 ± 2</td>
<td>19 ± 3</td>
</tr>
<tr>
<td>SCF</td>
<td>75 ± 2</td>
<td>7 ± 2</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>IL-6</td>
<td>70 ± 5</td>
<td>10 ± 3</td>
<td>20 ± 4</td>
</tr>
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</table>

*Fraction of D064 cells in different phases of the cell cycle after being cultured for 24 hours under various culture conditions; serum-containing medium (10% FCS) served as control. The amount of DNA per single cell was determined with PI staining and FACScan analysis. (Differences were not statistically significant).

permatant for up to 5% of the total culture volume) in the presence of 100 ng/mL of SCF resulted in optimum differentiation, whereas higher concentrations of IL-6 (added as supernatant for 10% or greater of the total volume) favored the proliferation of D064 cells.

Cell cycle analysis and viability of D064 cells. Cell cycle analysis of stromal cells showed that SCF reduced the number of cells in S-phase by 30%, in agreement with the observed low proliferation seen in the presence of SCF. In the presence of serum-containing medium or IL-6, 10%-12% of D064 cells were in S phase, 70% were in G1 phase, and 20% in G2 phase. In the presence of SCF, the cell cycle shifted towards G1 phase rather than G2 phase (Table 2). IL-6 had no visible influence on the cell cycle of D064 cells when compared with D064 cells grown in serum-containing medium. If no factors were added to the culture or cells were grown at low cell confluence, the number of apoptotic cells among D064 cells increased progressively until all cells died (Fig 7). Consistent with declining cell survival was the increased uptake of PI in D064 cells over time in serum- or factor-depleted tissue cultures (not shown).

DISCUSSION

We have shown that D064 cells proliferate or differentiate under the influence of well-defined soluble factors and less well-defined physical interactions. The differentiating non-adherent cells show increased expression of DR and exhibit features of hematopoietic precursor cells. However, at the early stages of differentiation the morphologic changes were reversible and cells could reattach and continue to proliferate in an adherent fashion. Dependent upon whether a positive or negative signal was received, transmitted or presented by the cells, D064 cells were directed towards differentiation or proliferation. The lack of a positive factor or contact signal led to cell death similar to the observations with neurons described by Raff et al.

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Intercellular communication between the cells is important for signaling and might trigger proliferation or differentiation or both. One relevant growth factor/receptor pair may be SCF and c-kit. The soluble, as well as the membrane-bound forms of SCF bind to c-kit, a tyrosine kinase receptor, which is not only expressed on hematopoietic cells, but at lower levels also on stromal and endothelial cells. The interactions between SCF and c-kit may contribute to the decision of stromal cells to differentiate or proliferate.

From a different viewpoint, the present results may have implications for the understanding of stromal cell physiology and biology. The maintenance of viability of stromal cells in culture depends on cell density and the presence of soluble factors. If the naturally occurring density of stromal cells is disrupted, the cells form clusters, which again increases the local density of cells as well as factors produced in an autocrine fashion. If stromal cells are not able to form clusters and maintain intercellular communication, they eventually undergo cell death. In conceptual agreement with this is the...
recent finding in a colon carcinoma cell line showing that apoptosis is induced by the inhibition of intercellular contact.\textsuperscript{28} In other models as well, cell survival depends on cell density and the secretion of survival factors.\textsuperscript{29}

One suitable candidate factor that has an effect on stromal cells in an autocrine or paracrine manner is SCF itself, which is mainly produced by stromal cells\textsuperscript{30,31} and exists in soluble and membrane-bound forms.\textsuperscript{32} The addition of exogenous SCF to D064 cells induced the differentiation of stromal cells.\textsuperscript{33} SCF also increased the expression of differentiation markers such as MHC class II (DR\textsuperscript{\*}) on stromal cells, an additional indication of differentiation.\textsuperscript{34} Interleukin-6 is another factor transcribed and expressed by stromal cells.\textsuperscript{35,36} IL-6 and SCF may have antagonistic activities with regard to stromal cell proliferation and differentiation as presented elsewhere.\textsuperscript{18} Both factors are also involved in the maintenance of viability of stromal cells, which otherwise undergo apoptosis. Apoptosis is a naturally occurring event among stromal cells, but its occurrence also depends on growth conditions. If stromal cells cannot interact with each other and no exogenous (eg, SCF) or autocrine factors (possibly also SCF) are present, the probability of apoptosis among stromal cells is increased. As stated above, the close cellular proximity between stromal cells (cluster formation) decreased the number of apoptotic events in culture. It has been shown by others that, especially under conditions of serum- or factor-depletion, the lack of intercellular contact ultimately leads to the death of cells.\textsuperscript{28} The ability of cells to proliferate or to avoid cell death is dependent not only on the number of cells in culture or the concentration of growth factors present,\textsuperscript{21} but also on the quality of the signals exchanged, which may be positive or negative.\textsuperscript{16,27}

In summary, multiple factors determine function and viability of the marrow-derived cell line D064. Viability is maintained in the absence of exogenous factors if cells are able to maintain contact or form clusters, presumably by interaction via membrane-receptors or by generating sufficient concentrations of autocrine factors. The addition of exogenous factors such as SCF and IL-6 enhances cell viability even at low cell concentrations; in addition, IL-6 stimulates proliferation, whereas SCF favors differentiation.

ACKNOWLEDGMENT

We thank Paul Goodwin for his help with the time-lapse video recording; Dr. James M. Roberts for discussion and suggestions on the cell cycle analysis; Judy Groombridge and Liz Caldwell for the manuscript; and D. Williams (Immunex Corp, Seattle, WA) and I. McNiece (Amgen Corp, Thousand Oaks, CA) for providing the various growth factors and for stimulating discussions.

REFERENCES

19. Radka SF, Charton DJ, Brodsky FM: Class II molecules of the major histocompatibility complex considered as differentiation markers. Hum Immunol 16:390, 1986
23. Zseo KM, Wypych J, McNiece IK, Lu HS, Smith KA, Kar-


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