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INTERACTION BETWEEN X-IRRADIATED PLATEAU-PHASE BONE MARROW STROMAL CELL LINES AND CO-CULTIVATED FACTOR-DEPENDENT CELL LINES LEADING TO LEUKEMOGENESIS IN VITRO

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Abstract

Plateau-phase mouse clonal bone marrow stromal cell lines D2XRII and C3H cl 11 produce decreasing levels of M-CSF (CSF-1), a specific macrophage progenitor cell humoral regulator, following x-irradiation in vitro. The decrease did not go below 40% of control levels, even after irradiation doses of 50,000 rad (500 Gy). In contrast, a distinct humoral regulator stimulating growth of GM-CSF/IL-3 factor-dependent (FD) hematopoietic progenitor cell lines was detected following radiation to doses above 2,000 rad. This humoral factor was not detectable in conditioned medium from irradiated cells, weakly detected using factor-dependent target cell populations in agar overlay, and was prominently detected by liquid co-cultivation of factor-dependent cells with irradiated stromal cell cultures. Subclonal lines of FD cells, derived after co-cultivation revealed karyotypic abnormalities and induced myeloblastic tumors in syngeneic mice. Five - eight weeks co-cultivation was required for induction of factor independence and malignancy and was associated with dense cell to cell contact between FD cells and stromal cells demonstrated by light and electron microscopy. Increases in hematopoietic to stromal cell surface area, total number of adherent cells per flask, total non-adherent cell colonies per flask, and cumulative non-adherent cell production were observed after irradiation. The present data may prove very relevant to an understanding of the cell to cell interactions during x-irradiation-induced leukemia.

Key words: Bone marrow stromal cells, x-irradiation, leukemia

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Introduction

Previous studies (1,3,8,9-11,13,19) have indicated that cell to cell contact between hematopoietic stem cells and stromal cells in continuous marrow culture is very relevant to the maintenance of hematopoiesis. Identification of specific junctional complexes between stromal and hemopoietic cell types has remained controversial but most groups have observed close membrane approximation between several cell types in the long term bone marrow culture system (3).

Several laboratories have reported permanent clonal cell lines derived from elements of the marrow stroma, with properties of fibroblast, reticular adventitial cell, endothelial cell, and adipocyte subtypes (12,26,29,31). These permanent clonal lines have been used to generate specific antibodies for cell surface determinants (31) and some have been shown to produce specific humoral regulators of hematopoiesis (23). Recent radiation biology studies with these clonal cell lines demonstrated radiation survival curves similar to that for fibroblast and epithelial cell types (5,6). The biology of irradiated plateau-phase cultures has recently become a subject of renewed interest, since the cells lining the marrow sinuses are normally in plateau phase and do not divide after irradiation of the marrow in situ (23).

We have recently reported an increase in proliferation of dual GM-CSF/IL-3 factor-dependent hematopoietic progenitor cell lines following co-cultivation with irradiated plateau-phase stromal cell cultures (24,25). Irradiation of stromal cells increased the total number of non-adherent factor-dependent cells produced by co-cultivation, and the emergence of a statistically significantly elevated number of factor-independent variant clonal sublines with distinct karyotypic abnormalities and tumorigenecity in vivo (24,25). In the present paper we document the light microscopic and ultrastructural properties of the cultures in which malignant transformation occurs.

Materials and Methods

Continuous Marrow Cultures

The methods for establishment of murine continuous bone marrow cultures have previously been reported (9,19). Briefly, the contents of an adult mouse femur and tibia were flushed via a 25 gauge needle into 24 cm^2 plastic Corning flasks in 25% horse serum-containing Fisher's medium, supplemented with 10^{-5}M hydrocortisone (9,19). All nonadherent cells and medium were removed weekly and cultures were maintained at 33°C, 7% CO_2.

Permanent clonal stromal cell lines

The derivation of the cell lines D2XRII and C3H/HeJ cl 11 have been reported (5,6,12,16,23, 24). The cells were passaged in Dulbecco's Modified Eagle's Medium, supplemented with 10% fetal calf serum. Plateau-phase cultures were established by transfer of $5x10^5$ cells to 25 cm^2 Corning plastic flasks in Fisher's medium supplemented with 25% fetal calf serum and 10^{-5}M hydrocortisone. Upon reaching confluence, plateau-phase cultures were maintained at 33°C, 7% CO_2 and media changed weekly. Under these conditions sloughing of adherent cell monolayers rarely occurred and the cultures were maintained in plateau phase for several weeks.

Derivation of factor-dependent cell lines

The multipotential hematopoietic progenitor cell line B6SUtA cl 5 has been reported (14). Cell lines bg/bg cl 1 and FDCP-1 have been reported (4,24). These prior 3 lines have been shown to be responsive to both GM-CSF and IL-3 (multi-CSF), but fail to divide in the absence of one of these two obligatory growth factors (17,20). The cell line 32D cl 3 has been shown to be uniquely responsive to IL-3 and fails to grow in GM-CSF (20). None of the above cell lines detectably grew in CSF-1 (M-CSF) (18), or in the absence of any added CSF (18). All cells were maintained in McCoy's 5A medium, modified as previously described and supplemented with 10% fetal calf serum and 10% dialyzed 5X concentrated WEHI-3 cell conditioned medium as a source of IL-3 (7,14,17). For co-cultivation studies described here, factor-dependent cells were washed in serum-free McCoy's 5A medium two times and then transferred to co-cultivation with control or irradiated plateau-phase stromal cells.

X-irradiation of stromal cell lines

Confluent plateau-phase cultures of D2XRII and C3H/HeJ cl 22 cells were x-irradiated by a 250 kVp orthovoltage unit as previously described, at a constant dose rate of 200 rad/min (11). Cultures were agar overlaid (as described below) immediately after irradiation, or were trypsinized and transferred into 35x10 mm tissue culture dishes (Falcon) at high density ($5x10^6$ cells per dish) to re-establish an adherent monolayer, and agar was overlaid on the following day. Another set of controls and 10,000 rad x-irradiated cultures was maintained at 33°C and cell-free conditioned medium was harvested weekly, and stored at -20°C.

Detection of colony stimulating factors (CSFs) in the conditioned medium

Fresh bone marrow, CBA mouse fetal liver cells or FDCP-1, 32D cl 3 and bg/bg cl 1 cell lines were washed 3 times in serum- free medium and resuspended in fresh medium with increasing concentrations of the CM harvested from control or irradiated D2XRII or cl 11 stromal cell cultures, containing 0.3% agar in 1 ml in 35x10 mm tissue culture dishes (Falcon). Colonies of $\geq$50 cells were scored on days 7, 14, and 21. Fresh marrow cells were obtained from C3H/HeJ mice on the day of the experiment. Single cells were similarly resuspended in the conditioned media containing 0.3% agar in 1 ml volume in 35x10 mm tissue culture dishes, and colonies scored on day 7. After scoring, the entire agar layer was removed, air dried on a glass slide and stained with Wright's Giemsa, mixed esterase stain or acetylcholine esterase stain (14).

For further morphological studies of the types of cells stimulated by each conditioned medium, CBA fetal liver cells were prepared in Dulbecco's modified Eagle's medium containing 20% heat-inactivated human plasma, 0.3% agar and 0.1 ml of the conditioned medium in 1 ml volume in 35x10 tissue culture dishes. Cultures were incubated at 37°C in a high-humidity incubator with 10% CO_2 and colonies of $\geq$50 cells were scored on day 7. To detect the possible ability of the conditioned media to initiate, but not sustain colony formation by other types of progenitor cells, replicate cultures were removed from the incubator after 2 days and 0.1 ml of pokeweed mitogen spleen conditioned medium (PM-SCM) was added as a source of IL-3 (18). After a further incubation period of 5 days, colonies of $\geq$50 cells were scored. After scoring, all the cultures were fixed *in situ* by adding 1.0 ml of 2.5% Luxol-fast-blue and counterstained with hematoxylin.

For the proliferation assay, $5x10^4$ cells of each line, 32D cl 3 and FDCP-1 were washed and resuspended in serial 10-fold dilutions of the conditioned medium harvested from control and x-irradiated D2XRII and clone 11 cultures in microwells (0.1 ml volume). After 24 h of incubation, the cells were pulsed with ^{3}H-thymidine (Amersham) and harvested 4 h later.

Cell-free conditioned medium from both stromal cell lines D2XRII and clone 11 was also tested for CSF-1 by radioimmunoassay (RIA) as previously described (28).

G-band karyotype preparation

Karyotyping was carried out according to published methods (10).

Tumorgenicity studies

Aliquots of $1x10^7$ or $1x10^6$ cells of each

cloned cell line were injected subcutaneously into the right flank of adult syngeneic C57BL/6J (for bg/bg cl 1) or B6D2F1 (for FDCP-1) mice. Mice were examined weekly for tumors at the site of injection. Dead or dying mice were subjected to complete autopsy according to published methods (12).

Results

Irradiation of plateau-phase bone marrow cultures was first carried out at varying doses between 2,000 and 20,000 rad. Under these conditions, no detectable alteration in morphology or saturation density was detected. Plateau-phase cells were maintained in culture at 33°C, 7% CO_2 for 4 weeks after irradiation, and no change was detectable compared to unirradiated cells. Co-cultivation of $5x10^5$ per flask cells of lines FDCP-1, bg/bg cl 1, B6SUtA, and 32D cl 3, was next carried out. There was no proliferation of 32D cl 3 cells under any of the irradiation conditions or using control unirradiated stromal cell cultures. In contrast, there was a low but detectable adherence and growth of cell lines bg/bg cl 1, B6SUtA, and FDCP-1 following co-cultivation with unirradiated D2XRII or cl 11 cells. Irradiation produced a dose-dependent increase in adherence proliferation and the percent of surface covered with co-cultivated factor-dependent cells.

The morphology of co-cultivation cultures in control and irradiated combinations is shown in Figs. 1-2. The ultrastructure of this interaction with individual cells is shown in Figs. 3-4. As Fig. 4 shows, there were numerous cell processes closely adherent between the hematopoietic cell interface with the irradiated stromal cell. In over 2000 individual cell to cell interfaces observed, this type of interface with numerous cytoplasmic processes was seen to be in association with stromal cells in the cultures.

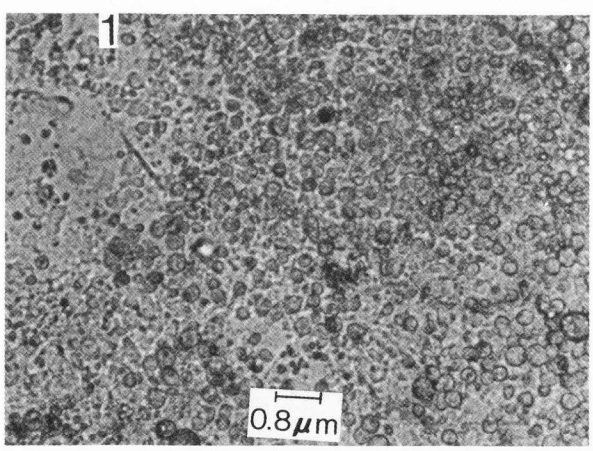

Figure 1: Morphology of FDCP-1 cells engrafted to 10,000 rad irradiated culture of D2XRII stromal cells at 7 days after graft

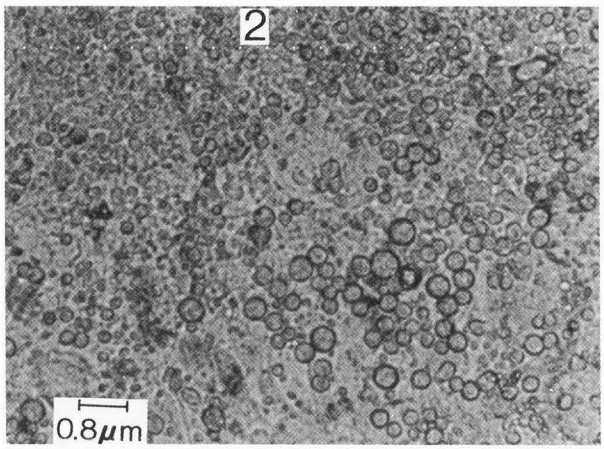

Figure 2: Morphology of the engrafted culture shown on Fig. 1 after 3 additional weeks

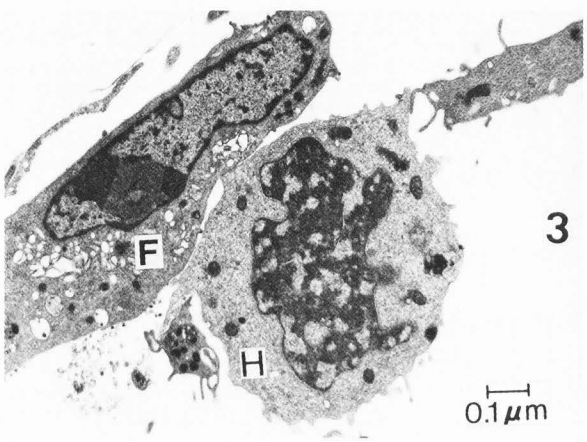

Figure 3: Transmission electron micrograph of interface between hematopoietic cell of line bg/bg cl 2 and irradiated D2XRII stromal cell. H=hemopoietic cell; F=fibroblast

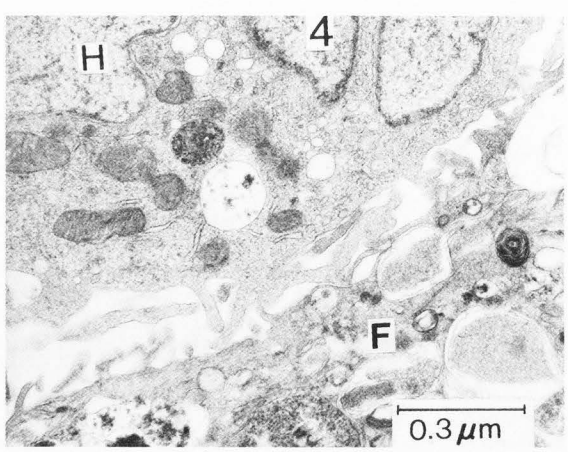

Figure 4: Higher magnification of interface between hematopoietic cell and 10,000 rad irradiated stromal cell. H and F as in Fig. 3

TABLE 1: Quantitation of "cobblestone"[a] islands of adherent GM-CSF/IL-3 dependent hematopoietic progenitor cell lines with x-irradiated cloned permanent stromal cell lines

Stromal cell line[a,d]	Factor-dependent cell line engrafted ($5x10^6$/ml) Adherent cobblestone islands per 30 mm^2 dish[b]							
	B6SUtA cl 3		32D cl 3		bg/bg cl 1		FDCP-1	
	day 1[c]	day 7	day 1	day 7	day 1	day 7	day 1	day 7
D2XRII	118±5	14±2	17±6	0	210±30	117±20	310±30	217±16
Clone 11	133±6	6±1	27±3	0	183±10	80±3	117±21	86±31
NIH/3T3	19±5	0	0	0	0	0	76±10	50±3
Balb/3T3	7±2	0	0	0	0	0	NT	NT
SFFV Balb 1902 B	23±6	1±0	0	0	0	0	NT	NT
mi/mi cl 1	86±2	10±2	10±2	3±1	17±3	3±1	17±6	3±1
mi/mi cl 4	14±3	0	NT	NT	14±3	8±2	8±2	0
mi/mi cl 3	0	0	NT	NT	NT	NT	8±1	0
mi/(+) cl 1	76±18	13±2	18±1	6±1	NT	NT	17±3	3
Sl/Sl[d] #1	0	0	NT	NT	0	0	0	0
Sl/Sl[d] #2	0	0	NT	NT	NT	NT	0	0
Sl(+/+) #1	73±2	10±2	6±1	3±1	NT	NT	16±2	8±1

[a] Cobblestone islands are each composed of ≥50 cells on stromal underlayers as described (8). Photos of cobblestone islands have been published (8), as have methods for scoring islands per flask (8).

[b] Stromal cell lines were derived according to published methods (12). The lines D2XRII and C3H/HeJd59 cl 11 (Clone 11) have been described (11). The cell lines mi/mi cl 1, cl 4, cl 3, mi(+) cl 1, Sl/Sl[d] #1, #2 and Sl(+/+) #1 have been described (16). The cell lines Balb/3T3, NIH/3T3 and SFFV Balb 1902B have been described (15).

[c] Days after engraftment of FD cells to stroma. Results are mean ± SEM for at least three dishes per point. NT = not tested

[d] References for these cell lines are delineated in previous publications (11,12, 15,16,24,25)

TABLE 2: Generation of factor independent tumor cell lines by co-culture of the cell lines with control or x-irradiated stromal cell cultures

	Derivation of F.I. clones harvested after co-culture for:			
	Wk 1	Wk 4	Wk 5	Wk 7
Stromal cell line Cl 11[1]				
Control unirradiated	0/10	0/15	0/15	0/50
10,000 rad irradiated	0/10	0/10	4/10	8/19
Stromal cell line D2XRII				
Control unirradiated	0/10	0/10	0/30	0/40
10,000 rad irradiated	0/10	0/10	3/19	10/10

[1] Cell lines Clone 11 or D2XRadII were grown to confluence in 30 mm^2 dishes as described in the legend to Table 1. Each was engrafted with $5x10^6$ FD cells of line bg/bg cl 3 at day 0, weekly thereafter all nonadherent cells were removed and plated 0.3% agar-containing culture at $1x10^6$ or $5x10^5$ cells/ml with no added source of IL-3 or GM-CSF. The harvested cells were then plated as single cells per 0.3 ml culture well. The number growing in medium without IL-3 or GM-CSF and establishing a subclonal permanent line is shown over the number of cells tested, at each week. Each factor independent (FI) line was tumorigenic in recipient C57BL/6J mice. (i.e. injection of $1x10^6$ cells subcutaneously into the flank of adult 30-33 gm mice produced solid ≥1 cm tumors by 6 months. No tumors were observed with the parent bg/bg cl 3 line or with cells harvested after 1 or 4 weeks co-cultivation.

Table 1 shows the adherent "cobblestone" areas (8) scored per flask at day 1 and day 7 after engraftment of each of 4 FD cell lines to each of 12 clonal stromal cell lines. Embryo fibroblast line NIH/3T3, Balb/3T3, SFFV Balb 1502B, and stromal cell lines Sl/Sl[d] #1 and #2 supported hemopoietic cell adherence and cobblestone island formation much less efficiently than did the other 7 stromal lines tested. Cell lines derived from C3H/HeJ mice (D2XRII and Clone 11) supported engraftment much better by these criteria than did other lines tested (Table 1).

Table 2 shows that removal of co-cultivated cells after irradiation of line D2XRII or Clone 11 led to the generation of factor-independent cell line variants at a significant level in several of the combinations. Karyotypic abnormalities and tumors following injection were not observed with any co-cultivation resulting with unirradiated stromal cell lines. In contrast, karyotypic abnormalities and tumors following injection of subclonal lines were observed with factor independent variants harvested after co-culture with unirradiated plateau-phase cultures (24). Thus co-cultivation lead to generation of factor-independent tumor cell lines in vitro.

Discussion

The mechanism of x-ray induced leukemic transformation of hematopoietic stem cells is not known. Most data argues that the mechanism is a direct effect on the hematopoietic stem cell (22, 30). This could be by x-ray induction of mutation, activation of oncogene or other specific cellular transforming gene products.

The present and recent (24) data provide the first body of evidence for an indirect x-ray induced transformation of hematopoietic stem cells through an initial effect on the stromal cells in the microenvironment. Several previous studies have indicated that ultraviolet light (21) and other toxic agents (2,26,27) can alter the mechanism by which one cell population interacts with another by altering the release of growth factors between cell membranes and by modulating the action of locally-reactive hormones. Further studies with this system may shed light on the mechanism of x-ray induced leukemic transformation.

Conclusions

The present studies indicate that x-irradiation of bone marrow stromal cells induces changes that are registered in a co-cultivated hematopoietic cell population. This data provides evidence that leukemogenesis by x-irradiation may be an indirect process involving a primary event in cells of the bone marrow stroma. While these data need to be confirmed by other studies, the possibility that x-irradiation leukemogenesis in fact is an indirect event. These data may have relevance for bone marrow transplantation in which leukemia recurrence in the donor cell population has been documented in several clinical studies. One interpretation of such clinical observations in the light of the present data is that donor hematopoietic stem cells co-cultivated with irradiated recipient stromal cells might have led to an indirect leukemogenesis through primary event in the host's stromal cell microenvironment.

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Discussion with Reviewers

Reviewer I: How did you ensure that the factor independent lines which you obtained after co-cultivation with the stromal lines were not of stromal origin?

Authors: The factor-independent lines were not of stromal origin because irradiated stromal cell cultures maintained and identically co-cultivated with dead factor-dependent cells produce no cell lines.

Reviewer I: Does the injection of control or irradiated stromal cells into adult or newborn mice cause tumors in these animals?

Authors: Introduction of control or irradiated stromal cells into adult or newborn mice does not cause tumors. These data are now out at 6 months and will be published at one year.

Reviewer I: Were the new factor independent lines that originated from co-cultivation with the stromal cells perhaps the results of endogenous viral activation? Was the supernatant from these lines tested for either reverse transcriptase activity or were there any viral particles detected by electron microscopy in either the supernatant or within the cells themselves?

Authors: New factor-independent lines originating from these cultures are not autocrine. The paper by Naparstek et al. (text ref. 23) demonstrates

this. Supernatant from the cell lines contains no detectable reverse transcriptase activity and no viral particles were detected by EM.

M. Tavassoli and C. Hardy: What is the effect of such a heavy dose of radiation on D2XRII cells? It is true that this is a plateau-phase culture, but does the radiation affect the subculture of this cell line? Does it have any effect on any aspect of cellular function?
Authors: Radiation effects on D2XRII cells have been described previously in the paper by Naparstek et al. (text ref. 23). The question of cellular function of these heavily irradiated plateau-phase cells is the subject of intense investigation and little data is available at the present time.

M. Tavassoli and C. Hardy: Can the tumor produced by the factor-independent cell lines be characterized as leukemia? Has this tumor been further characterized?
Authors: Tumors produced by factor-independent cells has now been characterized as myeloblastic fibroid tumors. We offer the reference, Naparstek et al. (text ref. 23) as further data.

M. Tavassoli and C. Hardy: Have the authors used those factor-dependent cell lines irradiated on long-term marrow culture instead of D2XRII cells and if so does it lead to factor-independency?
Authors: Factor-dependent cells co-cultivated with long-term marrow cultures convert to factor independence but at a lower frequency. Naparstek et al. (text ref. 24) substantiates this statement.