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Advances in Brief

Heterotransplantation of Early B-Lineage Acute Lymphoblastic Leukemia Using a Solubilized Attachment Matrix (Matrigel)[1]

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Abstract

Maintenance of long term culture and conventional xenografting of early B-lineage acute lymphoblastic leukemia cells is most difficult. Matrigel, a solubilized attachment matrix shown to aid growth of anchorage dependent solid tumors, was studied in heterotransplantation. Material for xenografting was derived from 5 patient bone marrow aspirates and 5 cell lines previously established and maintained by intraocular inoculation in nude mice. Specimens were injected by 3 methods: intraocular (n = 397); s.c. in medium (n = 78); and s.c. in medium supplemented by Matrigel (n = 69). With intraocular injection, 6 of 10 cell sources grew with respective ingraftment rates of 29–76%. Using the conventional s.c. method, no tumors resulted. The addition of Matrigel produced s.c. ingraftment from 8 of 10 cell sources (ingraftment rate, 50–100%). Immunophenotype, histopathology, and karyotype of the cells derived after Matrigel dependent ingraftment correlated with the cells of origin. It is concluded that Matrigel enables establishment and maintenance of early B-lineage acute lymphoblastic leukemia cell growth in a s.c. xenograft model.

Introduction

Early B-lineage ALL[3] is the most frequently occurring childhood neoplasm. A variety of methods have been attempted to propagate these cells in culture, and although short term clonogenic growth is possible (1) few have been effective in long term maintenance of cell lines (2). Heterotransplantation of patient material into immunodeficient animal models has potential advantages in both the propagation of human neoplastic cells and as a model for chemotherapeutic assays (3–5). However, ALL and, particularly the most common, early B-lineage subtype has been most difficult to xenograft using conventional methods (3, 6–10). We have previously described ingraftment and maintenance of ALL cells utilizing the immune privilege of the anterior segment of the nude mouse eye (4, 5). While this has allowed the establishment and propagation of a number of patient derived xenograft cell lines, there are limitations to this model. Matrigel has been reported to promote the growth of anchorage dependent cells in both tissue culture and xenograft systems (11–14). In this study we analyzed the potential benefits of Matrigel in the s.c. ingraftment of leukemia cells.

Materials and Methods

Cell Sources. All cells used in xenografting experiments were derived from patients at the Prince of Wales Children's Hospital, Sydney, Australia. Five of the 10 specimens were obtained directly from bone marrow specimens at either diagnosis or relapse. The 5 patients had early B-lineage ALL (1 multiphenotypic), with French-American-British classification L1 phenotype (1 mixed). Three were harvested at diagnosis and 2 were harvested at relapse. Bone marrow specimens were placed in Hanks' solution and separated by the Ficoll-Hypaque method. The resultant cells were washed and resuspended in RPMI 1640 at 5×10^6-1×10^7 cells/ml. The other 5 specimens were harvested from previously established i.o. xenograft cell lines (5). These cell lines had been previously characterized and the presence of Epstein-Barr virus DNA had been excluded by Southern blot analysis, using a probe containing an *Eco*RI Epstein-Barr virus fragment cloned in plasmid pACYC184 (5). Tumor filled eyes were enucleated under enflurane anesthesia (Ethrane; Abbott, Australasia Pty., Ltd.) and teased into suspension with 18-gauge needles, washed, and resuspended in the same concentration as above. Only viable cells were counted (trypan blue exclusion).

Experimental Animals. Nude mice (*nu/nu*) of BALB/c background (bred by the SPF Biological Facility, The University of New South Wales) were maintained in a protected and controlled environment (4). Sterile feed and water were provided as necessary and the cages were changed and sterilized weekly. Female mice 5–6 weeks of age and weighing 15–20 g were used. All procedures were carried out in a laminar flow hood, under anesthesia, in accordance with permits issued by the Committee on the use of Animals in Research or Teaching of the University of New South Wales, and guidelines of the National Health and Medical Research Council of Australia.

Heterotransplantation. A total of 544 aliquots from the 10 cell sources were injected using 1 of 3 methods: i.o.; conventional s.c.; and s.c. with Matrigel. The Matrigel (Collaborative Research, Inc., Bedford, MA) was supplied in 10-ml vials by Integrated Sciences Pty., Ltd., Sydney, Australia. Aliquots of 0.3 ml were kept frozen until usage and then thawed at 4°C and mixed undiluted with an equal volume of the cell suspension. Nude mice were anesthetized with enflurane, prior to being given bilateral i.o. or s.c. injections. For the s.c. cohorts volumes of 0.3 ml were injected with a 25-gauge needle, into the caudal flank regions containing either cell suspension or the mixture of Matrigel and cell suspension. For i.o. passage the eyes of anesthetized mice were held proptosed under a stereoscopic microscope (SV-6; Carl Zeiss, Germany) and an estimated 4–8-μl total volume of cell suspension was injected into the anterior segment through a 30-gauge needle attached to a 1-ml syringe. Ingraftment was determined by direct visualization of tumor growth in the s.c. sites or using the microscope for i.o. tumors.

Immunophenotyping. Immunophenotype was determined by fluorescence microscopy or flow cytometry. Monoclonal antibodies as listed in Table 2 were obtained from Coulter (Luton, Bedfordshire, England). Mononuclear cells were labeled with appropriately diluted antibody by incubation for 20 min at 4°C. After washing, cells were stained with fluorescein isothiocyanate-conjugated $F(ab')_2$ sheep anti-mouse immunoglobulin (Australian Monoclonal Development; Artarmon, New South Wales, Australia) followed by a second 20-min incubation. Cells were washed and analyzed using either an Olympus BH-2 fluorescent microscope (Olympus, Tokyo, Japan) or a FACScan (Becton-Dickinson, San Jose, CA). Dead cells were excluded from analysis on the basis of nuclear fluorescence.

Histopathology. The enucleated eyes were fixed in buffered formalin for 48 h and then sectioned sagittally to one side of the midline. The larger piece was processed following a further 24 h of fixation. The s.c. tumors were fixed in buffered formalin for 24 h and a representative section was processed. Both eyes and s.c. tumors were processed by dehydration through a series of graded alcohols, inbedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin.

Cytogenetics. Matrigel generated s.c. tumors derived from MMCL-4 and MS (Table 1) were analyzed cytogenetically. For the cell line MMCL-4, cytogenetic data were available from the i.o. xenograft cell source as well as

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[3] The abbreviations used are: ALL, acute lymphocytic leukemia; i.o., intraocular.

from 3 occasions during repeated Matrigel dependent s.c. passaging (2, 4, and 6 months from initial ingraftment). Chromosome harvest was carried out using standard techniques and classified according to the 1991 and 1985 International System of Human Cytogenetic Nomenclature. Briefly, 5×10^6 cells in suspension were harvested directly, and metaphase cells were arrested with 0.01 mg/ml Colcemid (Gibco) at 37°C for 45 min, exposed to 0.075 M KCl at 37°C for 15 min, and fixed in Carnoy's fixative. Following additional changes of fixes slides were prepared and air dried and the chromosomes were GTG banded. At least 30 karyotypes were analyzed in each of the tumors subjected to cytogenetic examination.

Results

Growth of Xenografts. In 8 of 10 cell sources s.c. inoculations with Matrigel produced measurable tumors (minimum 0.5 cm in diameter) in one or both flanks of the mice within a mean latency of 5.94 ± 0.49 (SE) weeks (Table 1). In consecutive passages the latency to tumor ingraftment decreased. To date the 8 lines have been maintained for up to 15 months through multiple s.c. passages (currently up to 9 passages). The s.c. inoculations without Matrigel were unsuccessful in all cases while i.o. ingraftment was achieved with 6 of 10 cell sources.

Table 1 *Ingraftment results*

Patient/cell line[a]	i.o. ingraftment	s.c. inoculation: No Matrigel	s.c. inoculation: Matrigel supplemented, Ingraftment	Mean latency, (wk) + *SE*
MMCL-4[1]	62/82 (76)[b]	0/12	16/17 (94)	4.56 ± 0.44
LMCL-5[1]	43/60 (72)	0/13	9/11 (82)	4.83 ± 0.40
LLCL-15[1]	46/69 (67)	0/12	3/5 (60)	6.33 ± 0.88
RMDL-13[1]	46/70 (66)	0/11	12/12 (100)	5.50 ± 0.67
LFCL-6[1]	54/78 (69)	0/18	2/4 (50)	11.00 ± 0.00
MS[2]-Dx	4/14 (29)	0/4	8/8 (100)	4.75 ± 0.48
TR[2]-Dx	0/4	0/2	5/6 (83)	9.67 ± 3.84
GK[2]-Dx	0/6	0/2	0/2	
NS[2]-Rel	0/6	0/2	0/2	
LC[2]-Rel	0/8	0/2	2/2 (100)	10.00 ± 0.00
	n = 397	*n* = 78	*n* = 69	

[a] Cell line nomenclature: patient initials (2 letters); abbreviated phenotype (3rd letter); L, leukemia (4th letter); digit, laboratory number; s.c., measurable tumor, minimum size, 0.5 cm diameter; i.o., visible tumor occupying minimum one-fourth of anterior segment. 1, previously established xenograft cell lines; 2, new patient material; Dx, sample derived at diagnosis; Rel, sample derived at relapse; *n* number of inoculations.
[b] Numbers in parentheses, percentage.

Immunophenotype. Sequential analyses by immunofluorescence indicated consistency through Matrigel dependent passage (Table 2). Subtle differences in phenotypic expression of individual antigens were observed (MMCL-4, LFCL-6, RMDL-13, and LC).

Histopathology. In 2 of the Matrigel dependent s.c. tumors (RMDL-13 and MMCL-4) histological comparisons were undertaken with the cells of origin (i.o. xenografts). In both instances the light microscopic patterns were unchanged and consistent with their lymphoid derivation (Fig. 1, *A–C*).

Cytogenetics. Clonal chromosome changes were identified in both the cell sources studied. Three related clones were identified in MMCL-4: 1 stemline karyotype, pseudodiploid, 46,XX,add(9)(p13); a mainline karyotype, 92,idemx2; and additional sideline 1, 92,idemx2, add(7)(p22). These karyotypic clones identified before injection with Matrigel remained unchanged through 6 months of Matrigel dependent passage as demonstrated at 2, 4, and 6 months. The karyotype of MS tumors was consistent with the hypodiploid subgroup of ALL: 45,XX,add(6)(q25),7,del(9)(p13) (15). The patient cells of origin were not successfully karyotyped.

Discussion

Current models for the continued propagation of ALL cells are less than satisfactory, particularly *in vivo* (1–10). More specifically, there are no reports of successful conventional xenografting from patients with early B-lineage ALL to nude mice. Matrigel has been introduced to enhance cell growth and differentiation of anchorage dependent cells from both normal and malignant tissues (11–14). Heterotransplantation of human solid tumors to nude mice has been reported to be enhanced by Matrigel (11–14). In many instances ingraftment could only be achieved with Matrigel (11, 14), while in others growth and differentiation were accelerated (12, 13). However, there has been no data regarding the role of Matrigel in the propagation of leukemic cells, either in cell culture or *in vivo*.

Table 2 *Immunophenotyping of patient and xenograft leukemia cells*

Patient/cell line[a]	B-lineage associated: HLA-DR	CD20	CD22	CD19	CD10	SIg	T-lineage: CD2	CD3	Myeloid: CD13 or CD33	TdT	Control
MM[P]	76				88	<1	<1		<1	8	
MM-CL4[IO]	3	10	<1	45	57	<1	<1		<2	3	<2
MM-CL4[M]	87	30	26	56	52	<2	<2		<2	3	<2
LM[P]	94				70	2	<5	4	<1		
LM-CL5[IO]				99	99	<1				91	8
LM-VL5[M]	99			95	99	<2	2		2	9	<2
LL[P]	76			97	(81)	1	<1	<1	<1	(90)	
LL-CL15[IO]	60			80	(83)	1	<1		<1	68	<1
LL-CL15[M]	97			92	(38)	<2	8		7	88	9
LF[P]	57			(31)	<1	(2)	2			(34)	<1
LF-CL6[IO]	88	3	1	(62)	54	<1					
LF-CL6[M]	4			2	9	(13)	2		2	2	<2
RM[P]	56	2	<1	50	86	<1	38			66	<1
RM-DL13[IO]	38	45	3	(41)	98	1	11			30	3
RM-DL13[M]	<1	<1	<1	84	1	<1	<1		<1	67	<1
MS[P]	58			85	64	<1	12	15	<1	<1	
MS[M]	94			98	85	<2	<2		<2	3	<2
TR[P]	82			97	94	<1	97	3	<1	98	
TR[M]	82			87	94	6	70	4	4	79	6
LC[P]	85			2	1	12	2	4	78	3	
LC[M]	47			14	24	4	16	11	89	<1	<2

[a] Cell line nomenclature: patient initials (2 letters); abbreviated phenotype (3rd letter); L, leukemia (4th letter); digit, laboratory number; P, patient of origin; IO, i.o. xenograft; M, s.c. xenograft with Matrigel. Quantitative immunofluorescence: numbers represent percentage positive. Qualitative immunofluorescence: numbers in parentheses, weak.

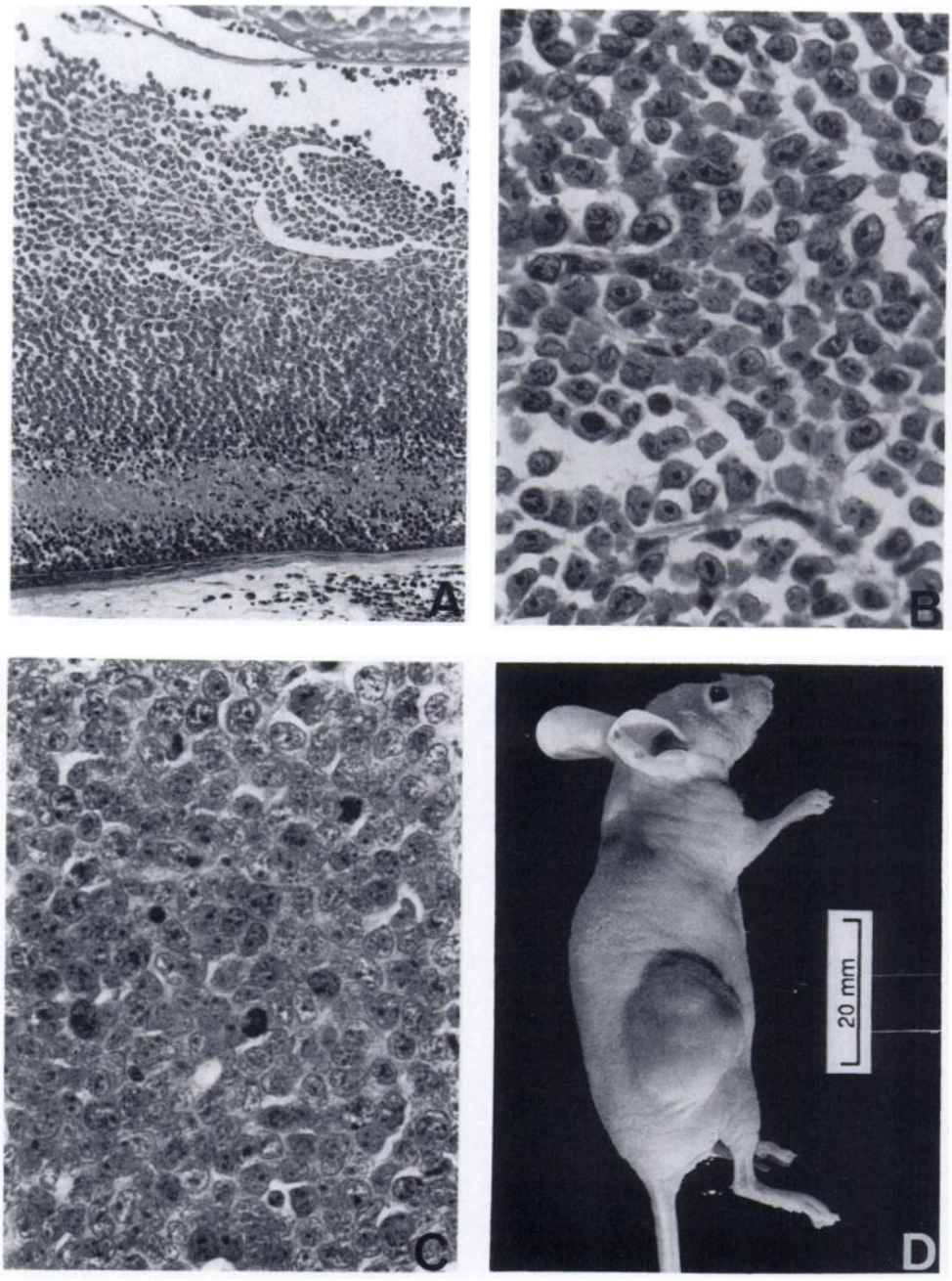

Fig. 1. *A*, Tumor cells (MMCL-4) in anterior segment of mouse eye (lens bottom of field). H & E, × 120. *B*, higher magnification of the same tumor in the anterior segment showing cells containing large, slightly irregular oval nuclei with vescicular chromatin, mainly a single prominent nucleolus and minimal cytoplasm, consistent with lymphoblasts. (*A* and *B* represent the source of the s.c. injection with Matrigel). H & E, × 495. *C*, s.c. tumor. Matrigel generated MMCL-4 s.c. tumor with cells very similar in appearance to those seen in *B*. H & E, × 495. *D*, nude mouse with Matrigel generated s.c. tumor.

In this study we have demonstrated Matrigel dependent s.c. ingraftment and maintenance of human early B-lineage ALL in nude mice. A total of 544 i.o. and s.c. sites were injected with leukemic cells. Ingraftment occurred in 255 of 397 i.o. injections (64%), in 57 of 69 s.c. injections with Matrigel (83%), and in none of 78 conventional s.c. injections. The failure of conventional s.c. ingraftment is consistent with our prior experience (4, 5) and other published data (3, 6–10). In particular, one would anticipate the greatest difficulty in xenografting early B-lineage ALL cells obtained from marrow specimens of patients at initial diagnosis. Two of the 3 cell sources obtained in this manner demonstrated s.c. ingraftment with Matrigel with rates of 100 and 83%, respectively. Furthermore, the cell numbers injected when the suspension was mixed with Matrigel were by definition halved as compared to the conventionally xenografted animals.

In order to enable propagation of leukemic cells as xenografts, several experimental models and immune manipulations have been attempted. These have included splenectomy, radiation, and other immunosuppression of nude mice (3, 6–10), the use of SCID mice (16, 17), and the injection into immune privileged sites (4, 5, 18). Each of these models have met obstacles in terms of either effectiveness or application. Heterotransplantation utilizing the anterior segment of the mouse eye has been a particular interest in our laboratory. This model has enabled both propagation and study of multiple leukemic phenotypes (4, 5). In the current experiments 5 of the 10 cell sources were previously established i.o. xenograft cell lines. The large numbers of i.o. injections in Table 1 include the data available from multiple prior passages of the respective cell lines. However, of the specimens derived from new patient material, only 1 of 5 sources produced i.o. ingraftment and this in only 4 of 14 aliquots (29%) (Table 1). This finding is consistent with our prior experience of relatively low success rates with early B-lineage cells obtained at diagnosis even in this otherwise highly effective model. The other limitation of the i.o. model has been the small cell numbers obtained from the end product. The Matrigel dependent s.c. tumor (Fig. 1*D*)

can provide an average of $5.7 \pm 1.4 \times 10^7$ cells by contrast with the $5.3 \pm 1.0 \times 10^6$ cells obtained from an infiltrated eye. Furthermore, in adapting the i.o. model for therapeutic assays there is the additional variable of drug penetration to the tumor site.

Although the mechanism of action of Matrigel is not fully understood, the current lines of investigation include structural (collagen mediated) enhanced cell to cell interaction and the presence of growth factors (19, 20). Whether any of these hypotheses are relevant to the demonstrated effect on leukemia xenografts remains to be considered. There are several potential benefits from the capacity to establish and maintain human leukemic cells in a nude mouse model that is both practical and quantitatively adequate. Furthermore, investigation of a potential role of Matrigel in cell culture of ALL may be of further interest.

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