Human leukemic T and B lymphoblasts produce insulin-like
growth factor binding proteins 2 and 4

E. Kirk Neely, Stephen D. Smith* and Ron G. Rosenfeld

Division of Pediatric Endocrinology, Stanford University,
and Division of Pediatric Hematology/Oncology*, University of Chicago, USA

Abstract. The production of insulin-like growth factors and
insulin-like growth factor binding proteins by twelve
human leukemic lymphoblast cell lines was evaluated by
radioimmunoassay, affinity cross-linking, ligand blot, and
immunoprecipitation of conditioned media. In all cell
lines, detectable IGF-I and IGF-II levels were <0.1 µg/l
and 0.3 µg/l, respectively. IGF binding proteins were
identified in 6/12 of the lymphoblast cell lines studied. A
pair of IGF binding proteins at 31 and 33 kD, immuno-
precipitated with an antibody recognizing IGF binding
protein 2 but not by an IGF binding protein 3 antibody,
was produced by both T and B cells. A 24 kD IGF binding
protein, presumably IGF binding protein 4, since it was
not recognized by the antibodies for IGF binding proteins
1, 2, and 3, was produced by B cell precursor cells and
faintly by one T cell line. These IGF binding proteins were
not altered by deglycosylation. Neither IGF binding
protein 1 nor IGF binding protein 3 was identified in any
of the conditioned media. We speculate that local pro-
duction of IGF binding proteins 2 and 4 regulates access
of the IGFBPs to lymphoblasts and to hematopoietic pre-
cursors in general.

Insulin and insulin-like growth factors I and II con-
stitute a family of related peptides with actions me-
chitivated by high-affinity receptors on the cell mem-
brane (1). The IGFs, but not insulin, also bind with
high-affinity to multiple IGF binding proteins
(IGFBPs) present in serum, other body fluids, and
the conditioned media of diverse cell lines (2-11).
Four human IGFBPs, with protein cores of approxi-
mately 25, 31, 29, and 26 kD, have been sequenced
(12-15) and have been termed hIGFBP-1, -2, -3,
and -4, respectively, and an additional IGFBP has
been identified by amino-terminal sequence (16).
Although it is known that hIGFBP-3, in its glyco-
sylated forms, is an important component of the 150
kD IGF serum carrier complex, and that IGFBPs
modulate access of IGFs to their receptors, their
regulatory role remains unclear (17-20).

We have previously demonstrated high levels of
insulin binding in B cell precursor acute lympho-
blastic leukemia (BCP-ALL or common acute lym-
phoblastic leukemia) cells and predominant IGF-I
binding in T lymphoblasts, a distinction which ap-
pears to correlate with insulin-stimulated prolifera-
tion of BCP-ALL cells and IGF-I stimulation of T
lymphoblasts in vitro (21,22). In the current study
we further investigate the role and regulation of
this peptide family in lymphoblasts by assaying for
IGF production and delineating the IGF binding
proteins produced by various B and T lymphoblast
cell lines.

Materials and Methods

Synthetic hIGF-II, provided by Dr C. H. Li (San Fran-
cisco, CA), was iodinated to a specific activity of 150-300
µCi/µg (11). Mouse monoclonal antibody 6303, recogniz-
ing IGFBP-1, was a gift of Dr E.-M. Rutanen (Helsinki)
(20). Rabbit polyclonal antibody alphaHecl, recognizing
hIGFBP-2 and hIGFBP-3, was raised against purified
media from the human endometrial adenocarcinoma cell
line Hecl 1A (8). A rabbit polyclonal antibody raised
against recombinant IGFBP-3 was kindly provided by Dr
V. Mukku (Genentech, S. San Francisco, CA).
Leukemic cell lines, derived from bone marrow samples at the time of diagnosis of children with acute lymphoblastic leukemia, were established and maintained as previously described (22). Patients were informed that samples would be utilized for research purposes, and approval was obtained from the Institutional Review Board. Cells were maintained in suspension in McCoy 5A medium (Irvine Scientific, Santa Ana, CA) with 15% fetal calf serum (FCS, Sigma, Grand Island, NY) at 37°C in a Hereaus incubator (Pleasant Hills, CA) gassed with 6% CO₂, 5% O₂, and 89% N₂. For conditioned medium (CM), cells were grown to 2 × 10⁶ cells/L, washed ×2 in serum-free McCoy 5A (SFM), and then maintained in SFM for 48 h. At harvest, supernatant was obtained from a 750 g spin and frozen at −20°C.

HepG2 human hepatoma cells, provided by Dr. A. D. Cooper (Stanford, CA), were maintained in RPMI 1640 (Irvine Scientific) with 10% FBS until confluent and then switched for 48 h to RPMI alone. Normal human adult fibroblast cell line N3652 (Human Genetic Mutant Cell Repository, Camden, NJ) was maintained in Dulbecco’s minimum essential medium (DMEM) (Irvine Scientific) plus 10% FBS and switched to serum-free DMEM (SFM) for 48 h. Cerebrospinal fluid was obtained from normal adult human volunteers.

Immunophenotype
Cell surface antigens of cell lines were identified by binding of monoclonal antibodies (Becton-Dickinson, Mountain View, CA), as detected by indirect immunofluorescence utilizing a fluorescence-activated cell sorter (FACS-IV, Becton-Dickinson).

Radioimmunoassay
Conditioned medium samples (10 ml) were chromatographed in 0.25 mol/l formic acid on a Sephadex G-50 column (Pharmacia, Piscataway, NJ). Peptide fractions were collected in 1% BSA, frozen and lyophilized, and subsequently evaluated by ligand blot for the presence of IGFBPs. IGF-I was measured using $^{[125]}$IGF-I as radioligand and polyclonal antisera UM487, a gift from NIDDK originally provided by Drs. J. J. Van Wyk and L. E. Underwood (Chapel Hill, NC). IGF-II RIA utilized $^{[125]}$IGF-II and a monoclonal anti-rat IGF-II antibody (Amano Enzyme, Troy, VA).

Affinity cross-linking
For cross-linking of CM, 20 μl samples were incubated at 23°C for 2 h with 50 000 cpm radioligand in 50 mmol/l TRIS at pH 7.4 for a total incubation volume of 50 μl (7). Proteins were cross-linked with 5 μl of disuccinimidyld isobutylate, the reaction quenched with 40 μl of 200 mmol/l TRIS HCl and 20 mmol/l EDTA, and the final volume brought to 128 μl by the addition of 33 μl of a sodium dodecyl sulphate (SDS) sample buffer (62.5 mmol/l TRIS, 10% glycerol, 2.35% SDS). Samples were run on a 12% SDS-polyacrylamide gel without reducing agents.

Ligand blot
Ligand blotting was performed as described previously (23). Samples of CM (105 μl) with 35 μl of SDS sample buffer were electrophoresed on 10% SDS-PAGE. Electrophoresed proteins were then electroblotted onto nitrocellulose filters using a Gelman Biotrans semi-dry electrophoretic transfer unit at 170 mAmp for 1.5 h. Filters were incubated overnight with 1.5 × 10⁶ cpm of $^{[125]}$IGF-II, washed and dried, and exposed to film for 10 days. Conditioned media electrophoresed and incubated with $^{[125]}$IGF-II exhibited no differences in IGFBP profiles from those incubated with $^{[125]}$IGF-I.

Results
$^{[125]}$IGF-II was cross-linked to CM from all of the cell lines. Fig. 1 presents the autoradiogram of cross-linked CM of T cells (lanes 1-6), B cell precursor (BCP-ALL/common ALL) cells (lanes 7-10), and Burkitt (mature B) cells (lanes 11, 12). In the CM of 3/6 T cells (SUP-T3, K-T1, SUP-T9) and 2/6 B cells (SUP-B27, SUP-B23), a 38 kD IGFBP band was present (31 kD after subtraction of the 7 kD mass of IGF). A broad 31 kD band (approximately 24 kD after subtraction of the 7 kD IGF) was seen in CM of 2/4 BCP-ALL cells (SUP-B2, SUP-B27),
Fig. 1. SDS-PAGE of [125I]IGF-II cross-linked to conditioned media from lymphoblastic cell lines: leukemic T cells (lanes 1-6), B cell precursor acute lymphoblastic leukemia cells (lanes 7-10), and Burkitt cells (lanes 11, 12).

but not in CM from mature B cells and only faintly in one T cell (SUP-T3).

CM from select cell lines which expressed IGFBP bands in cross-linking were subsequently analysed by ligand blot (Fig. 2). These CM samples from a T cell (K-T1), two BCP-ALL cells (SUP-B2, SUP-B27), and a Burkitt cell (SUP-B23) were also run after deglycosylation with Endoglycosidase F (even-numbered lanes). A 24 kD band that did not change with deglycosylation was seen in the CM of BCP-ALL cell lines SUP-B2 and SUP-B27 (lanes 3-4, 7-8) and was the same size as the 24 kD band in cerebrospinal fluid (CSF) and fibroblast CM.

This 24 kD IGFBP, presumably IGFBP-4, correlating to the 31 kD band seen on cross-linking, was not present in CM of K-T1 or SUP-B23. Two larger IGFBPs, a faint band at 33 kD and an intense band at 31 kD, were present in K-T1, SUP-B23, and SUP-B27 media, but only a light band at 31 kD was

Fig. 2. Ligand blot of IGF binding proteins in leukemic lymphoblast cell line conditioned media (CM), with (+) or without (−) Endoglycosidase F deglycosylation. CM samples ± Endoglycosidase F were separated by SDS-PAGE, transferred to nitrocellulose filter, and incubated with [125I]IGF-II. Lanes 1 and 2: T cell; lanes 3 and 4: B cell precursor acute lymphoblastic leukemia cell; lanes 5 and 6: Burkitt; lanes 7 and 8: BCP-ALL; lanes 9 and 10: cerebrospinal fluid (CSF); lanes 11 and 12: adult fibroblast CM; lanes 13 and 14, HepG2 CM.

Fig. 3. Ligand blots of conditioned media (CM) immunoprecipitated with antibodies 6303 (panel A) or alphaHecl (panel B) and incubated with [125I]IGF-II. Blots of CM alone (C), or immunoprecipitated with antibody (Ab) or non-immune serum (N), are shown for a T lymphoblast cell line (lanes 1-3), a B cell precursor cell line (lanes 4-6), and HepG2 or cerebrospinal fluid (CSF) (lanes 7-9).
seen in SUP-B2. Neither band of this pair, which correlates roughly to the 38 kD band seen on cross-linking, appeared to deglycosylate with Endo F. The intense 31 kD band migrated at the same size as the major CSF IGFBP, presumably IGFBP-2, which is also non-glycosylated (lanes 9, 10). The IGFBP bands at approximately 27 kD in CSF and HepG2 CM, and glycosylated IGFBP-3 at 37 and 42 kD in fibroblast CM (lanes 11, 12), were not seen in any of the lymphoblast CM. Similar results were seen with [125I]IGF-I.

In order to confirm the presence of IGFBP-2 and the absence of IGFBP-1 and IGFBP-3 in lymphoblast CM, immunoprecipitations were performed using antibody 6303 (Fig. 3A), recognizing IGFBP-1, and alphaHecl (Fig. 3B), which recognizes IGFBP-2 and IGFBP-3. In Panel A, the monoclonal antibody 6303 precipitated only the 26/28 kD doublet that is prominent in HepG2 CM (lanes 7, 8). None of the IGFBPs present in ligand blots of K-T1 and SUP-B27 was recognized by this antibody against IGFBP-1, AlphaHecl1 (Fig. 3B), which recognizes IGFBP-2, immunoprecipitated both components of the 33/31 kD doublet in K-T1 and SUP-B27 CM (lanes 2, 5) and the prominent 31 kD band in CSF (lane 8). A 28 kD band, of consistent density in all antibody lanes with alphaHecl, is artifactual, since it does not appear in ligand blots of CM alone and was present in a control lane of antibody with SFM only. The 27 kD IGFBP in CSF and the 24 kD IGFBP in SUP-B27 CM and CSF were not recognized by alphaHecl1.

Immunoprecipitation with antiserum generated against recombinant IGFBP-3 was performed in order to rule out the possibility that the 33/31 kD IGFBPs represent IGFBP-3, rather than IGFBP-2. In the ligand blot in Fig. 4, the 24 kD and 33/31 kD IGFBPs were visualized in the lanes of CM alone. Neither the 24 kD IGFBP nor the 33 and 31 kD IGFBPs was recognized by the anti-IGFBP-3 antibody (lanes 2, 5, 8, 11). In contrast, the prominent 37 and 42 kD bands in fibroblast CM were clearly immunoprecipitated with IGFBP-3 antibody (lane 14). The other fibroblast CM IGFBPs, at 31, 27, and 24 kD, were not immunoprecipitated.

All conditioned media were additionally assayed by radioimmunoassay for IGF-I and IGF-II. IGF-I levels were less than 0.1 μg/l in all samples. IGF-II levels were undetectable in 9/12 samples and less than 0.3 μg/l in the other 3 conditioned media. A BCP-ALL cell line with detectable IGF-II (SUP-B7) did not exhibit IGFBP in its conditioned media, while two T cells with detectable IGF-II production (K-T1 and SUP-T9) expressed IGFBP-2.

Table 1 and 2 summarize the monoclonal antibody profiles and IGFBP expression of the 6 T cell lines and 6 B cell lines. T cells (Table 1) are listed roughly from immature to mature, as CD-2, CD-5, and CD-7 are antigens appearing early in T cell differentiation, CD-4, CD-8, and CD-1 are intermediate, and CD-3 is a late, post-thymic marker. The three T cell lines which express IGFBP-2 may be classified as intermediate (SUP-T3, SUP-T9) or mature (K-T1), while the least mature T cell leu-

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<th>Cell line</th>
<th>CD-2</th>
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1Cells from lymphoblast cell lines were analysed utilizing various monoclonal antibodies as indicated by standard cluster determinant (CD) designation. IGFBP-2 or IGFBP-4 are listed as present if bands of the appropriate size were identified by either cross-linking or ligand blot.
kemia cell, SUP-T10, has not expressed IGFBP in several screening studies. Analogously, the B cells (Table 2) range from the CD-10+ (common ALL antigen+) early pre-B cells to the mature Burkitt cells expressing surface immunoglobulins. IGFBP-2 was visualized in CM of one B cell precursor and one Burkitt cell. In contrast, intense IGFBP-4 bands were seen in CM from two B cell precursor ALL cells and fainter bands were seen in CM of two other BCP-ALL cells, but not the Burkitt cells.

Discussion

From the available sequence data, at least five distinct IGF binding proteins exist (12-16). The four IGFBPs with completely deduced amino acid sequences are partially homologous and retain similar cysteine residues believed important for IGF binding. IGFBP-1, while prominent in human amniotic fluid and in Hepg2 conditioned media (5,6), apparently is absent in CM from many cultured cells. IGFBP-2, a major component of human cerebrospinal fluid and seminal plasma, is also present in human serum and in the CM of fibroblasts and breast and endometrial carcinoma cell lines (7-11). Studies in the rat indicate that it is a major IGFBP in fetal and newborn serum (24). IGFBP-3 is the principal IGFBP of adult human serum, forming a 150 kD complex with IGF and an acid labile subunit, and appears to be both growth hormone- and IGF-I dependent (2). IGFBP-4, seen in ligand blot of serum and many CM as a 24 kD band, has been sequenced from human bone cell conditioned medium (15). A fifth IGFBP aminoterminal sequence has been determined from an alternative IGFBP in human CSF resembling IGFBP-2 in size and higher affinity for IGF-II (16).

By cross-linking, ligand blot, and immunoprecipitation techniques, employing either [125I]IGF-1 or [125I]IGF-II, only two IGFBPs were present in the conditioned media of T and B lymphoblasts. In ligand blots, 37-42 kD bands representing IGFBP-3 were completely absent, and the 29 kD deglycosylated IGFBP-3 was not present in any of the CM after Endo F treatment. Furthermore, immunoprecipitation with an antibody against recombinant IGFBP-3 failed to demonstrate any immunoreactive IGFBP. In a similar manner, ligand blots of CM and immunoprecipitation with monoclonal antibody 6303 failed to demonstrate IGFBP-1. Additionally, lymphoblast CM did not contain the glycosylated 28 kD IGFBP that is present in fibroblast CM (but is distinct from IGFBP-1, which is non-glycosylated).

On the other hand, IGFBP-2 could be identified in CM of select T and B cells. In several ligand blots, a faint band was seen at approximately 33 kD and an intense band at 31 kD, the latter comigrating with presumed IGFBP-2 in CSF. Like IGFBP-2, neither IGFBP of the pair was deglycosylated by Endo F. Both bands were immunoprecipitated with alphaHec1, which recognizes both IGFBP-2 and IGFBP-3. Because neither band was recognized by the specific IGFBP-3 antibody, we conclude that both bands represent IGFBP-2. However, while alphaHec1 does not recognize IGFBP-1 or IGFBP-4, its specificity is not completely defined, and the minor 33 kD band might represent another IGFBP altogether. A candidate for migration at 33 kD might be the IGFBP reported from human CSF as similar in size to, but distinct from, IGFBP-2 (16).
Alternatively, although unlikely, a partially deglycosylated IGFBP-3, which can also be seen at this size in fibroblast CM after Endo F treatment, might be present in lymphoblast CM, yet not be recognized by the specific IGFBP-3 antibody.

We presume the 24 kD IGFBP to be identical with IGFBP-4 recently sequenced by Ling and colleagues (15). An IGFBP of comparable size is seen in CSF and fibroblast CM and has previously been noted also in serum, seminal plasma, human breast cancer cell CM, and other CM (4,7-11). It is of interest in this study that only B cell precursor ALL cells produced substantial amounts of this IGFBP. However, production of IGFBP-4 is not a phenomenon of B cells exclusively, since a faint IGFBP-4 band was seen in cross-linking of CM from a T cell line.

With the possible exception of IGFBP-4 production only in immature leukemic lymphoblasts, principally in BCP-ALL cells, there appears to be little relationship between IGFBPs and cell lineage. The most immature T cell (SUP-T10) did not express IGFBP, but at least some of the immature B cells secreted both IGFBP-2 and -4. Additionally, IGFBP secretion appears not to be regulated by local IGF production, since T and B lymphoblast cell lines, including those exhibiting IGFBPs, expressed negligible levels of both IGF-I and IGF-II.

Neither IGFBP-1 nor IGFBP-3 was identified in any of the conditioned media. Furthermore, neither IGFBP-1 nor IGFBP-3 was expressed after stimulation of selected cells by IGF-I, IGF-II, or insulin (unpublished observations). The absence from lymphoblast CM of IGFBP-3, so prominent in the CM of many other cells (in contrast with IGFBP-1, which is expressed by a smaller proportion of cell lines), may have an important physiologic significance that is not clearly understood at this time. Recently, protease activity affecting IGFBP-3 has been identified in human serum during pregnancy (25). Analysis of CM from several lymphoblast cell lines has not, however, revealed any protease activity that contributes to the absence of IGFBP-3 in these cells (unpublished observations).

A mitogenic response to the IGFs has been demonstrated in lymphoid, myeloid, and erythroid precursors (26-28), providing a putative role for IGFs in hematopoiesis. The production by lymphoblasts of IGFBP-2 and IGFBP-4, but not of IGFBP-3, suggests a mechanism for local regulation of IGF access to lymphoid precursors and hematopoietic precursors in general. The lymphoblast cell lines that did not produce any of the IGFBPs are excellent candidates for further approaches to the regulatory role of IGFBPs in IGF-responsive cells. Because it has been demonstrated that growth of T lymphoblast cell lines is stimulated by IGF-I (22), assay of IGF action in these non-IGFBP-producing lines, either in the presence of exogenous purified IGFBPs or after transfection with IGFBP cDNA, provides a promising experimental system to investigate the role of IGFBPs in IGF action.

It has also been reported that growth hormone stimulates growth of transformed T cells via production of IGF-I and a postulated autocrine action (29). The role of GH and IGFs in lymphoid cells is of particular interest to endocrinologists not only because of the reported, but controversial association of GH treatment and leukemia, but also due to the increasing number of individuals referred following successful treatment of leukemia by various modalities (30,31). The potential effects of GH, or indeed of IGF therapy, on normal and neoplastic bone marrow components warrant further experimental attention to the role of IGFs and their binding proteins in lymphoid and myeloid precursors.

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Dr E. K. Neely,
Department of Pediatrics, S 322,
Stanford University Medical Center,
Stanford,
California 94305,
USA.