The Proto-Oncogene c-fgr Is Expressed in Normal Mantle Zone B Lymphocytes and Is Developmentally Regulated During Myelomonocytic Differentiation In Vivo

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The proto-oncoprotein c-fgr is a member of the c-src gene family of nonreceptor protein tyrosine kinases. This gene family currently has nine members: c-src, c-fgr, c-yes, lyn, lck, lyn, blk, hck, and yrk. The expression of the src gene family members has been extensively studied. Several members including c-src, yes, lyn, and lyn are expressed in a wide variety of cell types, whereas c-fgr, lck, hck, and blk have a more restricted pattern of expression in hematopoietic cells. The oncogene c-fgr is expressed in normal human neutrophils, monocytes, macrophages, and natural killer cells. Data from the in vitro differentiation of normal bone marrow-derived monocytic cells and several myelomonocytic cell lines suggest that c-fgr is developmentally regulated. Specifically, c-fgr expression is induced as these cells acquire a more mature phenotype. Data from several studies of c-fgr expression in myeloid leukemias further support this observation with c-fgr expression in leukemic blasts correlating with a more mature myeloid/myeloid phenotype. It has not been previously determined whether c-fgr is regulated in a similar fashion during normal myelomonocytic differentiation in vivo.

The normal biologic functions of c-fgr are not currently known. The protein p55fgr has been shown to physically associate with the FcγRII receptor in human neutrophils and with Ly6C in a murine monocytic cell line. The contribution of p55fgr to the signal transduction pathways of these receptors is unclear. The targeted disruption of c-fgr in mice has recently been reported. These mice have normal hematopoiesis. Macrophages isolated from these mice are phenotypically and functionally normal in a variety of in vitro tests. Further, c-fgr mutant mice have an apparently normal immune response to Listeria monocytogenes, an intracellular pathogen. However, mice with targeted disruptions of both c-fgr and Hck (but not Hck alone) have an increased susceptibility to Listeria monocytogenes. These data indicate that c-fgr plays a (as yet undefined) role in the immune response to this pathogen.

The oncogenic potential of the src gene family is well established. v-fgr, v-src, and v-yes were originally identified from acutely transforming retroviruses isolated from naturally occurring tumors. In addition, several of the protooncogenes, including c-fgr, are transforming in NIH 3T3 assays. Finally, the lck protooncogene has been shown to cause thymic tumors in transgenic mice when overexpressed. There has been little data directly linking the src gene family with human neoplastic disease. Expression of c-fgr has been detected in the B cells of chronic lymphocytic leukemia (CLL) patients and in Epstein-Barr virus (EBV)-associated lymphoproliferative disorders. Of note, c-fgr expression has not been detected in normal or reactive human lymphoid tissue. The possible deregulation of c-fgr in these lymphoproliferative disorders suggests that the c-fgr protooncogene may play an important role in the pathogenesis of these diseases.

This study was designed to carefully examine normal human hematopoietic and lymphoid tissues for c-fgr expression using immunohistochomical techniques to detect p55fgr at the single-cell level. We show here that c-fgr is developmentally regulated during normal myelomonocytic maturation with high-level p55fgr expression beginning during the myelocyte stage of development. In addition, we show that p55fgr expression is readily detectable in B cells isolated from patients with CLL but not in normal circulating B lymphocytes. Interestingly, examination of lymphoid tissues demonstrated that p55fgr is also expressed in a normal B cell subset, the mantle zone B lymphocytes.
MATERIALS AND METHODS.

Production and purification of c-fgr antiserum. The region of the human c-fgr gene encoding for amino acids 1 through 88 was subcloned into the prokaryotic expression vector, pET 3a (Novagen, Madison, WI). Recombinant c-fgr peptide was produced in Escherichia coli, as described, and the expected 9-kD peptide was purified by electrophoresis through a 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Gel slices containing the isolated c-fgr peptide were used to immunize NZW rabbits. The murine monoclonal antibodies used in this study have well-defined tissue specificities and were used at the manufacturers’ (DAKO, Carpinteria, CA, for L26, KP-1, and UCHL-1; Clonab, Denville, NJ, for LN-2) recommended dilution: L26 (1:200), KP-1 (1:800), UCHL-1 (1:400), and LN2 (1:20). For the immunohistochemical studies, a biotinylated goat anti-rabbit IgG (Sigma) and alkaline phosphatase-conjugated streptavidin complex (Gibco BRL, Gaithersburg, MD) were used for detection. The specimens were treated with alkaline phosphatase substrate kit II (Vector Laboratories, Burlingame, CA) and counterstained with methyl green and alacian blue. For the immunofluorescence studies, the specimens were incubated with phocyerythrin-conjugated goat anti-rabbit and fluorescein-conjugated goat anti-mouse F(ab)2 fragments (TAGO Immunologicals, Camarillo, CA). Where indicated in the text, the c-fgr antiserum was preincubated with a molar excess of either GST::fgr or GST alone for 1 hour at 4°C.

Lymphoid tissue was obtained from material submitted to the Department of Pathology, Washington University School of Medicine (St. Louis, MO). The tissue was embedded in OCT compound (Miles Laboratories, Elkhart, IN), snap-frozen in liquid-cold isopentane, and stored at −70°C. The histologic diagnosis was based on conventional hematoxylin-stained, formalin-fixed, paraffin-embedded sections. Frozen sections were cut (6 μ), fixed briefly in ethanol, and rehydrated in PBS. Immunostaining studies were performed on these specimens as outlined above.

RESULTS.

c-fgr antiserum characterization. The region of the human c-fgr gene encoding amino acids 1 through 88 was cloned into the prokaryotic expression vector pET 3a, and recombinant c-fgr peptide was isolated as described in Materials and Methods. This peptide was used to immunize rabbits, and the resulting antiserum was affinity purified over a glutathione-Sepharose column (see Materials and Methods). The specificity of this antiserum was tested with immunoblots and cell staining. HeLa cell extracts containing the indicated src kinase listed in Fig 1 were blotted in duplicate and probed with either an antiphosphotyrosine antibody (Fig 1A) or the c-fgr antiserum (Fig 1B). Expression of the appropriate sized protein is apparent in each extract, but only p55fgr/56hck is detected with the c-fgr antiserum.

Similar experiments were performed to ensure that the c-fgr antiserum did not detect btk protein. Another src-related protein, btk is primarily expressed in B lymphocytes. Cell extracts were prepared from WEHI-231 cells that express abundant btk but no detectable c-fgr and from HL60 cells treated with retinoic acid, which express abundant c-fgr. These extracts were blotted in duplicate and hybridized with either anti-btk antiserum (data not shown) or the c-fgr antiserum (Fig 1C). The p50hck protein is present in the WEHI-231 extract, as expected (data not shown). The c-fgr antiserum readily detects p55fgr/56hck in the HL60 cell extract but does not crosshybridize with p50hck.

The proto-oncogenes hck and c-fgr have similar patterns of expression. To test the ability of the c-fgr antiserum to discriminate between p55fgr and p56hck, K562 cell lines were generated that expressed human c-fgr, human hck, or vector alone. Approximately equivalent amounts of p55fgr and p56hck were detected by immunoblotting with the relevant antiserum (data not shown). These cell lines were
Fig 1. Specificity of c-fgr antiserum: Western blots. (A) Antiphosphotyrosine blot. (B) Anti-c-fgr blot. Cell lysates from HeLa cells overexpressing the indicated src-related kinase were blotted in duplicate and hybridized with the indicated antiserum. The appropriate sized protein is readily detected for each src gene family member with the antiphosphotyrosine antiserum. In contrast, only p55 is detected with the c-fgr antiserum. (C) Anti-c-fgr blot of cell lysates prepared from WEHI-231 or HL60 cells induced with retinoic acid. The c-fgr antiserum does not crossreact with p50 present in the WEHI-231 cell extract.
C-fgr expression in normal B lymphocytes. Several recent reports suggested that C-fgr is not expressed in normal human B lymphocytes. We used our C-fgr antiserum to examine p55<sup>CR</sup> expression at the single-cell level in normal B-lymphocyte populations. Frozen sections obtained from normal and reactive lymph node tissues were stained with the C-fgr antiserum; representative results are shown in Fig 4. Moderately intense C-fgr staining was seen in the mantle zone of the follicle, while the germinal center cells were mostly negative (Fig 4A and C). Occasional positive cells were also detected in the interfollicular areas. Similar results were obtained with reactive lymph node (data not shown).

To determine the lineage of the cells expressing p55<sup>CR</sup>, dual immunofluorescent techniques were used. Frozen sections of normal lymph node tissue were stained with the C-fgr antiserum and with one of the following antibodies: LN-2 (a B lymphocyte-specific marker that preferentially stains mantle zone B lymphocytes), UCHL-1 (a T lymphocyte-specific marker), or KP-1 (a macrophage/monocyte-specific marker). Representative results are shown in Fig 5. LN-2 and C-fgr double-positive cells are seen in the mantle zone, demonstrating that p55<sup>CR</sup> is expressed in mantle zone B lymphocytes. Note that no C-fgr staining is again seen in

stained with the C-fgr antiserum, as shown in Fig 2. K562 cells expressing p55<sup>CR</sup> stained strongly (Fig 2C), whereas cell lines expressing vector alone or p56<sup>hck</sup> (Fig 2A and B, respectively) showed only background staining. In addition, preincubation of the C-fgr antiserum with the GST-fgr fusion protein abolished the cellular staining (Fig 2D). Collectively, these data demonstrate the specificity of this antiserum.

c-fgr expression in human bone marrow cells. Mononuclear cells were purified from normal bone marrow aspirates and stained with the C-fgr antibody. Representative results are shown in Fig 3. The p55<sup>CR</sup> protein was most strongly detected in myelocytes, metamyelocytes, and more mature myeloid cells. Promyelocytes stained less intensely. Cells of erythroid lineage did not stain. The expression of C-fgr in normal blast cells was difficult to ascertain because of the limited number of identifiable blasts in these cell preparations. These data confirm that C-fgr is predominantly expressed in myelomonocytic cells and suggest that the highest level of expression within the myeloid lineage is seen in terminally differentiated myelomonocytic cells.

c-fgr is expressed in a subset of normal B lymphocytes. Several recent reports suggested that C-fgr is not expressed

Fig 2. Specificity of C-fgr antiserum: Immunohistochemistry (original magnification, × 1,000). K562 cells derived from clones expressing vector alone (A), human hck (B), or human C-fgr (C and D) were stained with the C-fgr antiserum. (D) The C-fgr antiserum was preabsorbed with a GST-fgr fusion protein. The C-fgr protein is readily detected in the C-fgr-expressing cells, but only background staining is seen with vector alone- or human hck-expressing cells. Preincubation of the C-fgr antiserum with the GST-fgr fusion protein blocks specific staining.
Fig 3. Cell c-fgr staining in human bone marrow cells (original magnification, ×1,000). Normal human bone marrow cells were stained with the c-fgr-specific antibody (A and B). (C) The antiserum was preabsorbed with the GST-fgr fusion protein. The c-fgr protein is readily detected in cells with morphology consistent with myelocytes (large arrow), metamyelocytes (small arrow), and more mature myeloid forms (large arrow heads). Promyelocytes (small arrow heads) contained little detectable c-fgr protein.

Fig 4. Staining of c-fgr in a human lymphoid follicle. Lymphoid follicle from a normal human tonsil stained with the c-fgr-specific antiserum with (B) or without (A and C) preincubation with GST-fgr fusion protein. (A) The mantle zone B lymphocytes show moderate staining for c-fgr, whereas the germinal center B lymphocytes showed no staining for c-fgr. Occasional cells in the interfollicular areas also stained for c-fgr (original magnification, ×100). (C) Higher magnification of mantle zone-germinal center junction. Mantle zone B lymphocytes (arrow) show moderate c-fgr staining (original magnification, ×600). (B) No staining is seen with c-fgr antiserum preabsorbed with GST-fgr fusion protein.

gern cell center cells. Intensely staining c-fgr and KP-1 double-positive cells were seen scattered throughout the interfollicular and mantle zone areas (Fig 5C); these cells most likely represent tissue macrophages. No c-fgr and UCHL-1 double-positive cells were detected (data not shown), indicating that p55c-fgr is not expressed in interfollicular T lymphocytes. These results demonstrate that p55c-fgr is expressed in a discreet subset of normal B lymphocytes, the mantle zone B lymphocytes.

c-fgr is expressed in circulating B lymphocytes isolated from CLL, but not normal, patients. Previous reports suggested that c-fgr mRNA is expressed in CLL B cells. To confirm these findings and to ascertain whether p55c-fgr is also expressed in normal circulating B lymphocytes, we examined peripheral blood mononuclear cells from either normal subjects or five patients with CLL. The specimens were stained with the c-fgr antiserum and L26, a B lymphocytespecific marker. Representative results are shown in Fig 6. In contrast to normal circulating B lymphocytes (Fig 6A), CLL B cells contain readily detectable amounts of p55c-fgr (Fig 6B). Note that p55c-fgr is expressed in peripheral blood monocytes, as expected.
DISCUSSION

Previous studies of c-fgr expression in normal human tissues have used RNA analyses of tissue specimens that contained cells of several different lineages. Data from these studies suggested that c-fgr was primarily expressed in mature myelomonocytic cells, namely, neutrophils, monocytes, and macrophages. No c-fgr mRNA was detected in normal or reactive lymphoid tissue. In this study we have determined the pattern of expression of p55<sup>c-fgr</sup> in hematopoietic and lymphoid tissues at the single-cell level using immunostaining techniques. This approach has enabled us to definitively identify the p55<sup>c-fgr</sup>-expressing cells within a mixed cell population.

In vitro differentiation of murine bone marrow-derived monocytes and myelomonocytic cell lines is associated with an increase in c-fgr expression. Furthermore, c-fgr expression in leukemic blasts is associated with a more differentiated myeloid or monocylic phenotype. Our data demonstrate that p55<sup>c-fgr</sup> is also induced during normal myeloid differentiation in vivo. High-level p55<sup>c-fgr</sup> expression is first seen during the myelocyte stage of development and persists through the mature neutrophil stage. The myelocyte stage...
of myeloid differentiation is notable for the acquisition of secondary granules and for the loss of proliferative capacity as maturation continues to the metamyelocyte stage. It is interesting to speculate that p55-src may play a role in the terminal differentiation of neutrophils. A similar function has been postulated for p56lck during T-cell development.32

As noted above, previous studies have detected little or no c-fgr expression in human lymphoid tissues. In the current study, we have detected moderate p55-src levels in mantle zone B lymphocytes. In contrast, no p55-src was detected in germinal center or peripheral blood B lymphocytes. The observed difference in p55-src expression between the current study and previous studies is most likely secondary to the ability of immunostaining techniques to detect p55-src in a minor cell type within an otherwise negative mixed cell population. In agreement with our data, Hatakeyama et al recently reported that p55-src is expressed at high levels in murine lymph node, although the identification of p55-src-expressing cells within the lymph node was not performed in this study.

Mantle zone B lymphocytes are small resting B lymphocytes.33 In contrast to germinal center B lymphocytes, mantle zone B lymphocytes are capable of secreting immunoglobulin in response to pokeweed mitogen.31 Phenotypically, mantle zone B lymphocytes most closely resemble peripheral blood B lymphocytes, with surface expression of IgM, IgD, CD20, CD21, and Leu-8 antigens.34,35 The precise role that mantle zone B lymphocytes play in the immune response, as well as the biologic significance of p55-src expression in these cells, is not currently known. Expression of several other members of the src gene family has been detected in B lymphocytes, including p56lck, p53/56 fos, p50f, and p50fr. In particular, p56lck expression has been detected in the dense lymphocyte fraction obtained from gradient centrifugation of tonsillar lymphocytes;30 this fraction is known to contain the mantle zone lymphocytes.36 Several members of the src gene family have been implicated in the signal transduction pathway of the IgM receptor complex.37,38 It may be that c-fgr serves a similar function for a B-lymphocyte receptor that functions in this restricted B-lymphocyte subpopulation. It would be interesting to examine mice with the targeted disruption of the c-fgr gene for abnormalities in their humoral immune response.

A consistent finding in EBV-infected B-cell lines and EBV-immortalized B-lymphoblastoid lines is the expression of c-fgr mRNA and protein at levels comparable with that seen in normal monocytes or neutrophils.18,39 We have shown that c-fgr transcripts in EBV-positive B cells arise from a promoter that is distinct from that used in myelomonocytic cells.40,41 The contribution of EBV to the regulation of c-fgr expression in B cells is controversial. Sharp et al42 reported that the EBV-negative B-cell line Ramos had higher c-fgr mRNA levels than several EBV-positive B-cell lines. In addition, they demonstrated that c-fgr expression was downregulated in EBV-positive B-cell lines with α-interferon treatment. Their data suggested that c-fgr expression was not directly regulated by EBV. In contrast, Knutson et al43 reported that acute infection of primary B lymphocytes or EBV-negative B-cell lines with EBV was associated with an increase in c-fgr mRNA levels. Furthermore, she reported that the EBNA-2 gene of EBV was sufficient to upregulate c-fgr expression in an EBV-negative B-cell line. Our laboratory has been unable to reproduce these results; we have found no consistent elevation of c-fgr mRNA levels with either acute EBV infection or EBNA-2 expression in B cells (Link DC, Sugden B, and Ley TJ, unpublished observations, May 1991). In the current study, we demonstrate that p55-src is expressed in a normal B-lymphocyte subset. This observation suggests that c-fgr expression in B cells is regulated by a specific B-cell genetic program. Collectively, these data suggest that c-fgr expression is indirectly regulated by EBV; EBV may, under certain circumstances, trigger a specific B-cell program that leads to c-fgr expression.

Achts et al44 reported a survey of c-fgr expression in various lymphoid malignancies. They found that all 22 of the CLL-derived samples had readily detectable c-fgr mRNA, whereas normal circulating B lymphocytes had no detectable c-fgr mRNA. This study did not, however, exclude contaminating monocytes from the CLL B-cell preparations; these monocytes may have contributed significantly to the c-fgr mRNA detected in these preparations. To avoid this problem, we examined p55-src expression in CLL B cells at the single-cell level. Our findings show that, indeed, p55-src is expressed in the circulating B cells of patients with CLL. Further, we have detected p55-src expression in tumor specimens of patients with well-differentiated lymphocytic lymphoma, a B-cell disorder closely related to CLL (Link and Zutter, unpublished observations, January 1994).

The pathogenesis of CLL at a molecular level is poorly understood. Evidence for the disregulation of the c-fgr proto-oncogene in these B cells might suggest an important role for c-fgr in the pathogenesis of CLL. However, the demonstration that c-fgr is expressed in a normal B-lymphocyte subpopulation suggests that its expression in this lymphoproliferative disorder may result from the activation of a specific B-cell genetic program. Its expression in these neoplastic B cells may, therefore, be an indirect consequence of, rather than a primary cause of, the neoplastic transformation process.

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