Differential Expression of Id Genes in Multipotent Myeloid Progenitor Cells: Id-1 is Induced by Early- and Late-Acting Cytokines While Id-2 is Selectively Induced by Cytokines That Drive Terminal Granulocytic Differentiation

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Abstract Hematopoietic development is regulated by a complex mixture of cytokine growth factors that guide growth and differentiation of progenitor cell populations at different stages in their development. The genetic programs that drive this process are controlled at the molecular level by the type and number of transcriptional regulators coexpressed in the cell. Both positive- and negative-acting helix-loop-helix transcription factors are expressed during hematopoietic development, with the Id-type transdominant negative regulators controlling the net helix-loop-helix activation potential in the cell at any given time. It has been demonstrated that some of these Id factors are involved in the checkpoint at which undifferentiated progenitor cells make the commitment to terminal maturation. Therefore, we sought to determine whether these Id family factors are selectively induced or extinguished by cytokines that act at different points during hematopoiesis. NFS-60, a myeloid progenitor line that proliferates in response to multiple cytokines, was stimulated by treatment with SCF, IL-3, IL-6, G-CSF, and erythropoietin. Id-1 expression correlated tightly with cellular proliferation: it declined when growth factor stimulation was withdrawn and was quickly induced whenever the cell began to proliferate. The regulation of Id-2 was more complex: its expression was slightly upregulated in factor-deprived cells but only strongly reinduced after extended exposure to cytokines that drive granulocytic differentiation (IL-6, G-CSF, and TGFβ1). These data support a cell-cycle regulatory role for Id-1 in multipotent myeloid progenitor cells and a role for Id-2 during terminal granulocytic differentiation. J. Cell. Biochem. 71:277–285, 1998. © 1998 Wiley-Liss, Inc.

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Differentiation of hematopoietic cells is controlled both by factors driving cellular proliferation as well as by induction of terminal differentiation gene programs. This multistage process is guided by the complex mixture of cytokines produced in the bone marrow. Some of these cytokines are known to be important for the growth of early progenitors, others are essential for lineage-specific terminal differentiation, and other cytokines contribute to hematopoietic development at multiple points. Individual cytokines may regulate the cell cycle machinery, activation of genes necessary for function of the mature blood cell, or both. All of these processes are regulated at the molecular level by coordinated expression of lineage- and stage-specific genes.

The helix-loop-helix (HLH) family of transcriptional regulators play an important role during development in many organ systems. Members of this family have been shown to be important factors for regulation of neural development [Caudy et al., 1988; Nagata and Todokoro, 1994], sex determination [Caudy et al., 1988], and myogenesis [Olson, 1992; Wein-

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traub, 1993] as well as contribute to regulation of development in the pancreas [Nelson et al., 1990], bone [Ogata and Noda, 1991; Park and Walker, 1992], and fatty tissue [Tontonoz et al., 1993]. There is evidence that this family contributes to the control of hematopoietic development as well [Aplan et al., 1992; Begley et al., 1989; Cooper et al., 1997; Goldfarb and Lawandowska, 1995; Kuo et al., 1991; Massari et al., 1998; Shoji et al., 1994; Visvader et al., 1991; Zhuang et al., 1994].

The function of HLH transcriptional regulators is dictated by three structural elements: 1) the helix-loop-helix protein dimerization interface, 2) a basic, DNA binding region, and 3) an N-terminal transactivation domain. The HLH structure is a protein dimerization motif that is used to combine HLH monomer subunits into functionally important dimers [Murre et al., 1989]. Upstream and extending from helix one there is usually a basic region, which is a DNA binding half-site. When these proteins dimerize they juxtapose the two half-sites into an active DNA-binding element that can then recognize and bind to specific sequence motifs called E-boxes. Finally, in some of these monomer subunits, there is an N-terminal transactivation domain that effects transcriptional activation once the active dimer has been localized to its correct position in a promoter or enhancer regulatory unit [Aplan et al., 1990; Shoji et al., 1994; Weintraub et al., 1991].

There are variants that function as negative regulators as well. In some instances, alternative splicing generates HLH monomers lacking the transactivation region: heterodimerization between this monomer and an full-length HLH activator would be expected to decrease or extinguish the activation potential of the resultant dimer [Aplan et al., 1990]. Another subclass of HLH forms completely lack the basic, DNA binding region. These proteins make up the Id (*I*nhibitor of *D*NA binding) family, which can heterodimerize with bHLH members to form dimers containing an incomplete DNA binding region. These dimers cannot then bind to DNA [Benezra et al., 1990; Christy et al., 1991; Riechmann et al., 1994; Sun et al., 1991]. Thus the Id family proteins inhibit transcriptional activation by sequestration of the HLH activators in nonfunctional complexes. The Id-type transdominant negative regulators of this family are highly expressed in progenitor cells of many types but generally are extinguished as cells terminally differentiate. Experimental overexpression of these Id genes creates a block to differentiation, even in the presence of the sufficient differentiative stimuli.

Since cytokines guide hematopoietic progenitor cells into suitable pathways for terminal differentiation, it is of interest to determine whether these same cytokines have a direct effect on expression of Id-type HLH regulators. To explore this possibility, we used the murine promyeloblast cell line, NFS-60. These cells are blocked in their ability to differentiate but can respond by proliferation to a wide variety of cytokines important for hematopoietic development [Holmes et al., 1985; Weinstein et al., 1986]. Using this system, we showed that Id-1 expression correlated tightly with cellular proliferation. The regulation of Id-2 is more complex: its expression was slightly increased in factor-deprived cells but only strongly induced after extended exposure to cytokines that drive granulocytic differentiation (IL-6, G-CSF, and TGFβ1).

MATERIALS AND METHODS

Cell Culture

NFS-60 cells were routinely cultured in RPMI 1640 plus 10% Fetal Calf Serum (FCS) and 10% WEHI-3B conditioned medium (WEHI cm). For the cytokine response studies, cells were washed three times in RPMI plus 5% heat-inactivated FCS, then resuspended at low concentration (0.1×10^6/ml) in the same medium. Cells were cultured in the absence of exogenous cytokines for 18 h treatment with recombinant cytokines at concentrations previously determined to give maximal growth stimulus: Interleukin-3 (IL-3, Collaborative Research, Inc., Bedford, MA) at 100 U/ml; Interleukin-6 (IL-6, R & D Systems, Minneapolis, MN) at 50 ng/ml; G-CSF (R & D Systems) at 1000 U/ml; GM-CSF (Amgen Biologicals, Thousand Oaks, CA) at 100 U/ml; Stem Cell Factor (SCF, R & D Systems) at 50 U/ml; erythropoietin (Epo, Toyobo, Osaka, Japan) at 5 U/ml and TGFβ1 (R & D Systems) at 100 ng/ml. In the experiments with IL-3, G-CSF, and TGFβ1 in combination, cells were treated with 100 ng/ml TGFβ1 or sub-saturating concentrations of 1 U IL-3 and 5 U G-CSF per ml. Aliquots of cells were counted for number and viability at 0, 3, 8, and 24 h (for the data presented in Figs. 1–3) or 0, 2, 8, 24, and 48 h (for the data presented in Fig. 4) then processed for mRNA isolation. All experiments were per-

formed at least twice; data shown in each figure is from a single, representative experiment.

RNA Preparation and Analysis

RNA was isolated from pools of 10^7 cells using an mRNA isolation kit (Pharmacia, Gaithersburg, MD) and 1 mg of polyadenylated RNA was analyzed by Northern blot. Blots were hybridized to high specific activity Id-2 cDNA probes, then stripped and probed with Id-1 then GAPDH. Id-3 and Id-4 are not expressed in NFS-60 cells and were not analyzed. Expression was quantified by phosphorimage analysis (Molecular Dynamics, Sunnyvale, CA) and individual values normalized for loading efficiency with GAPDH. Filters hybridized to Id-2 were exposed to film for 10 days (5 days on the phosphor screen), Id-1 was exposed for 3 days (24 h on the phosphor screen), and GAPDH was exposed for 18 h (3 h on the phosphor screen). In each case, films were overexposed to maximize visualization of faint bands.

RESULTS AND DISCUSSION

Differentiation of hematopoietic cells is controlled at the molecular level by coordinated expression of lineage- and stage-specific genes. Both positive- and negative-acting HLH regulators are coexpressed during this process, with the Id-type transdominant negative regulators controlling the net HLH activation potential in the cell at any given time. It has been demonstrated that some of these Id factors are involved in the checkpoint at which undifferentiated progenitor cells make the commitment to terminal differentiation. Since cytokines guide hematopoietic progenitor cells into terminal differentiation, we wished to determine whether these cytokines could mediate some or all of that effect through the Id-type HLH regulators.

Id-1 expression correlates directly with proliferation in NFS-60 cells. Id-1 expression is high in logarithmically-growing NFS-60 cells but dropped approximately four-fold after cytokines have been withdrawn for 18 h. At this point, NFS-60 cells remain viable (80–100%) but are no longer proliferating. We have assayed the levels of Id-1 gene expression 3, 8, and 24 h after introduction of individual cytokines to the growth-arrested cells. NFS-60 cells proliferated strongly in response to treatment with IL-3, IL-6, G-CSF, Epo, and SCF but did not proliferate when treated with medium alone or with GM-CSF (Fig. 1). Expression of Id-1 correlated well with periods of proliferation, being high in the starting population and strongly reinduced after addition of IL-3, IL-6, G-CSF, Epo, or SCF (Fig. 2). Thus this activity does not have apparent correlation with signaling through any cytokine receptor subfamily and hence for any discrete signaling pathway; any cytokine that stimulated proliferation also induced expression of Id-1. It is formally possible that the recombinant cytokines have acted synergistically with trace amounts of cytokines from the 5% heat-inactivated serum or through induction of secondary cytokine expression in NFS-60 cells. However, these data clearly show a causal relationship between treatment of these cells with individual cytokines and activation of Id-1 (and Id-2, below) gene expression.

SCF alone does not play a role in terminal differentiation of hematopoietic cells [Broudy, 1997] whereas IL-3, IL-6, G-CSF, and Epo are all known to be involved in mediation of terminal granulocyte, monocyte, and erythroid differentiation, respectively [Nicola, 1989]. However, when NFS-60 cells were treated with any of these cytokines, the cells proliferated strongly and did not undergo differentiation detectable by histologic analysis (Wright-Giemsa staining, [Weinstein et al., 1986] and data not shown).

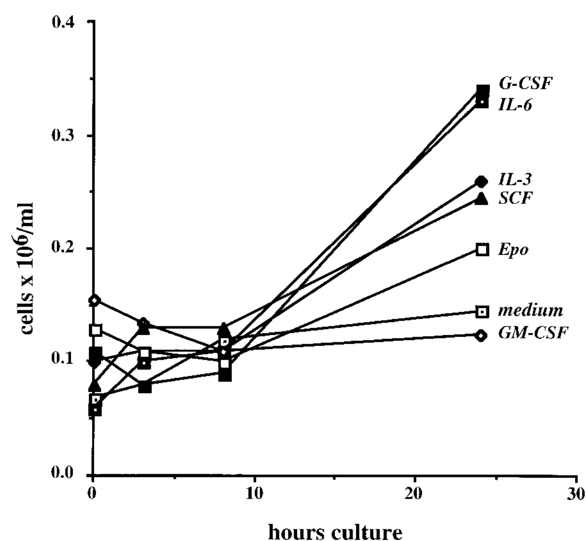
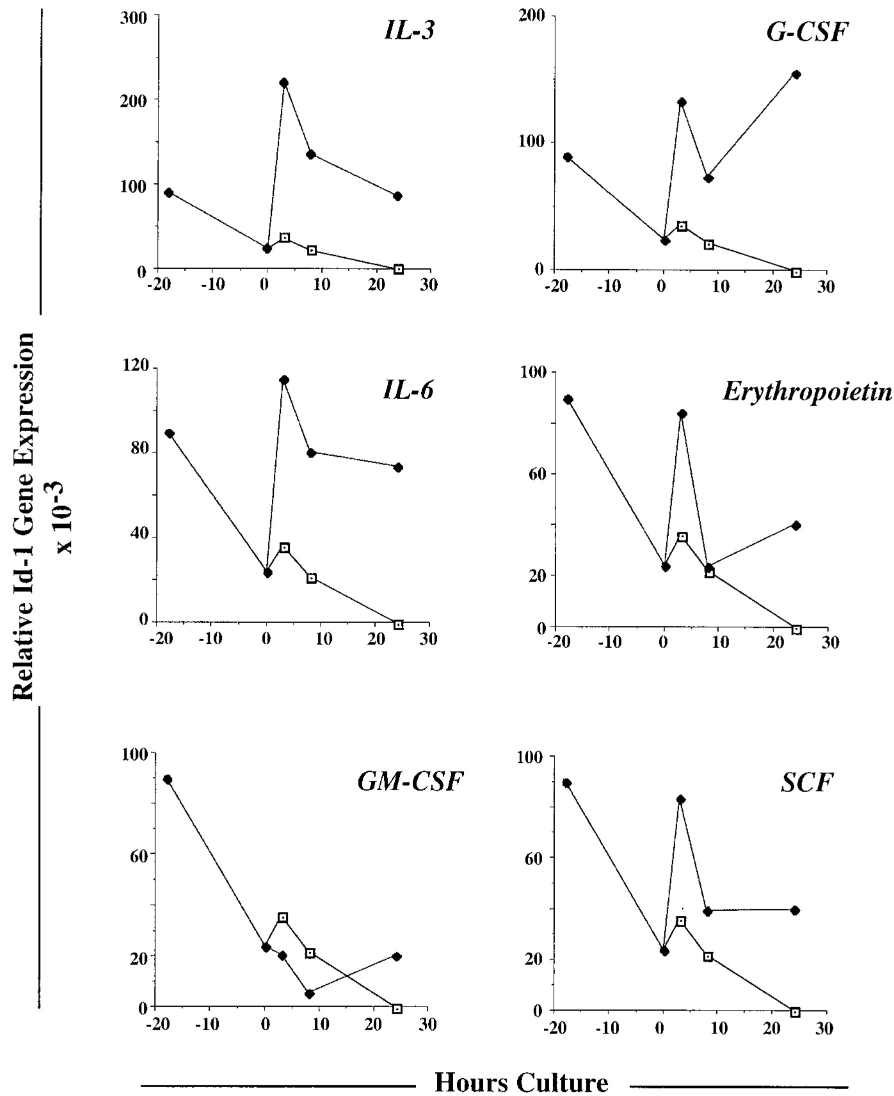


Fig. 1. Growth of NFS-60 cells after treatment with hematopoietic cytokines. NFS-60 cells were starved of cytokines for 18 h, then saturating amounts of recombinant cytokines were added and the cells grown for 24 h. As a control, cells were continuously incubated in medium plus 5% heat-inactivated FBS alone. Proliferation is presented as number of viable cells/ml culture medium.

A.



B.

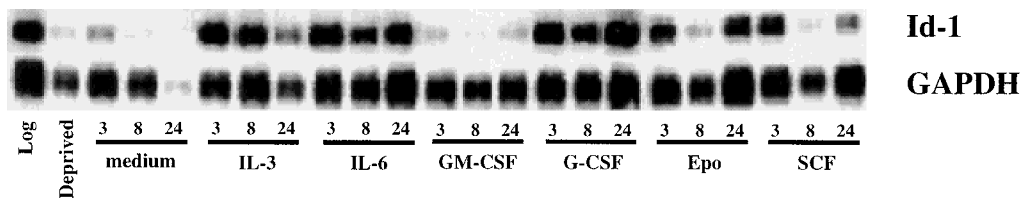
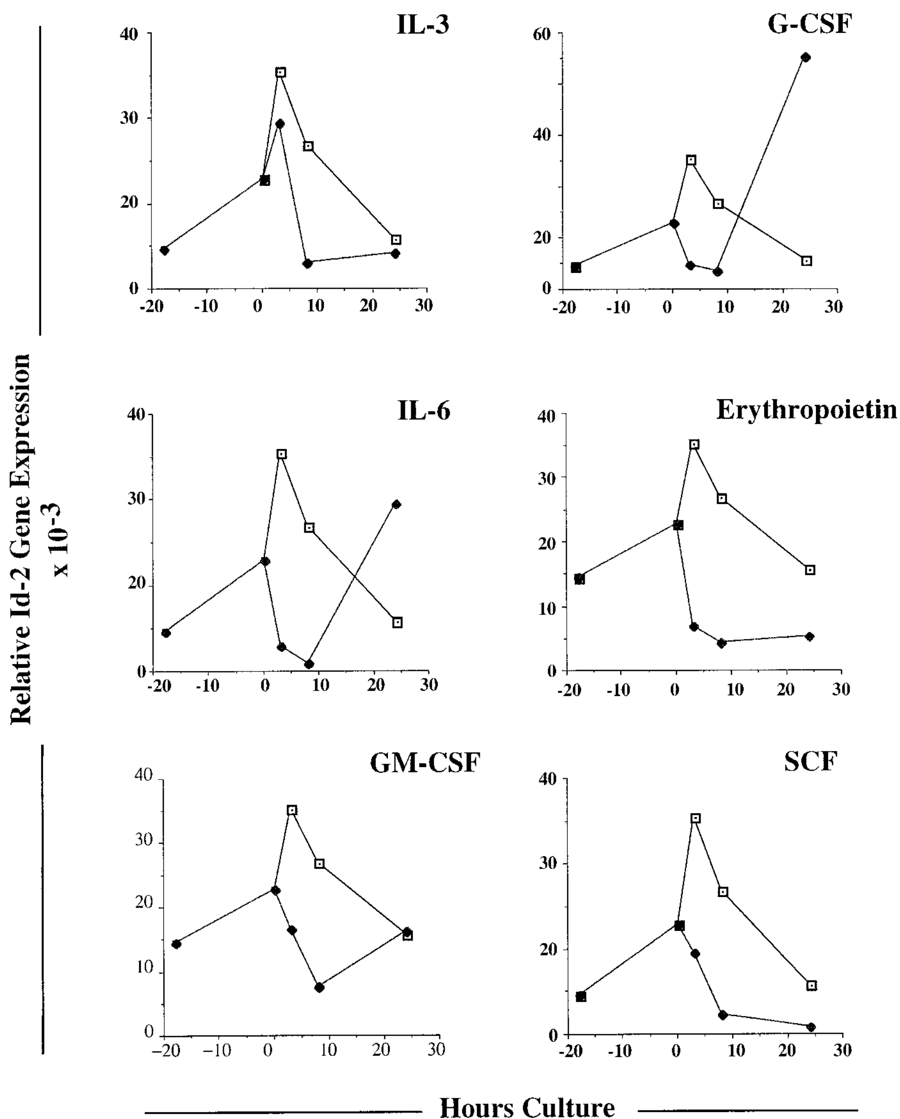


Fig. 2. Id-1 gene expression correlates with cell proliferation. **A**: mRNA was harvested from cytokine-stimulated cells and the level of Id-1 gene expression determined by northern hybridization to cDNA probes. Levels of Id-1 gene expression in cells continuously incubated in medium alone are graphed with open boxes; Id-1 gene expression after cytokine treatment is graphed with closed diamonds. Blots were quantified by phosphorimage analysis and the individual values adjusted for loading efficiency by normalization to GAPDH expression. Data from a representative experiment is presented. **B**: Raw data is presented from scanned autoradiographs.

A.



B.

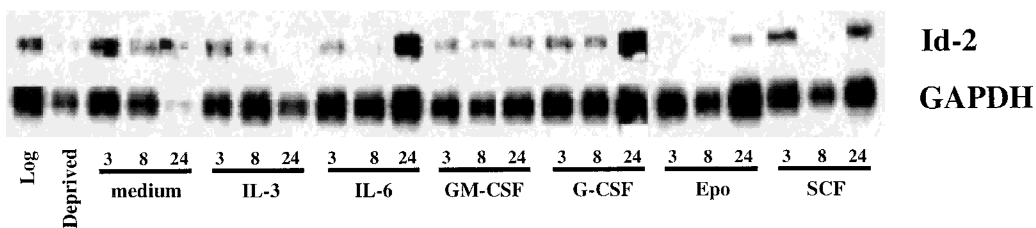


Fig. 3. Id-2 gene expression is elevated by cytokines that play stimulate granulocytic development. **A**: Blots used in Figure 1 were stripped and reprobed with ^{32}P-random-primed Id-2 cDNA. Levels of Id-2 gene expression in cells continuously incubated in medium alone are graphed with open boxes; Id-2 gene expression after cytokine treatment is graphed with closed diamonds. Data from a representative experiment is presented. **B**: Raw data is presented from scanned autoradiographs.

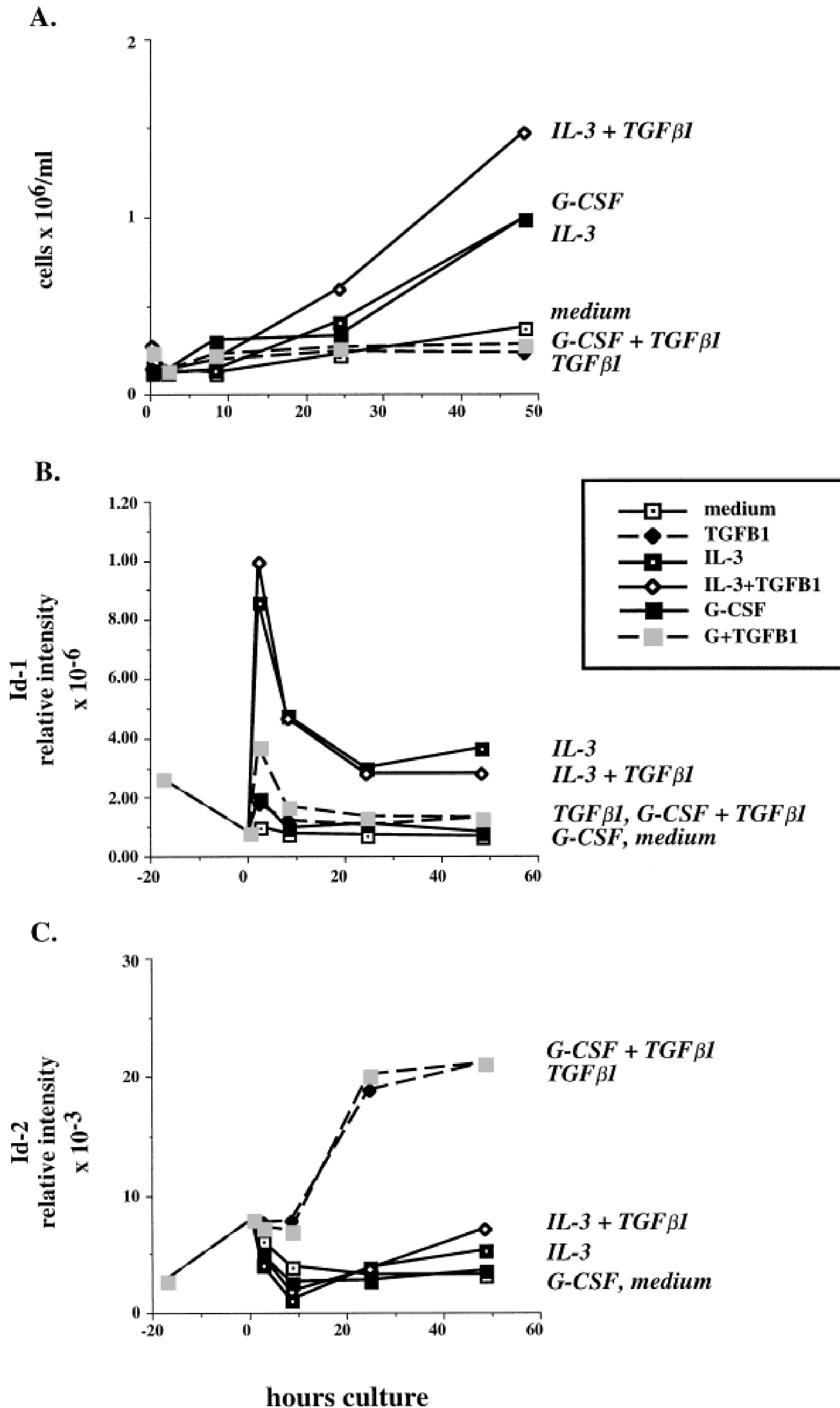


Fig. 4. TGFβ1, a strong stimulant for neutrophil development, induces Id-2 expression in NFS-60 cells. NFS-60 cells were starved of cytokines for 18 h, then subsaturating amounts of IL-3 (1 U/ml) G-CSF (5 U/ml), or TGFβ1 (100 ng/ml) were added alone or in combination, then the cells were grown for 48 h. As a control, cells were continuously incubated in medium plus 5% heat-inactivated FBS alone. **A**: Proliferation of cytokine-treated NFS-60 cells, presented as number of viable cells/ml culture medium. **B,C**: mRNA was harvested at the time points indicated and the level of Id-1 (B) and Id-2 (C) gene expression determined by northern hybridization to cDNA probes. Blots were quantified by phosphorimage analysis and the individual values adjusted for loading efficiency by normalization to GAPDH expression. Data from a representative experiment is presented.

Id-1 gene expression levels were elevated, with variable kinetics, in all proliferating cells.

NFS-60 cells, like normal progenitors of similar developmental stage, are completely dependent upon cytokines for growth and survival [Weinstein et al., 1986]. However, this cell line is transformed by the Cas-Br-M murine leukemia virus, which has integrated into the 3' end of the sixth exon of the c-*myb* locus [Weinstein et al., 1986]. Constitutive overexpression of c-*myb* has been demonstrated to block terminal differentiation in hematopoietic cells [Clarke et al., 1988; Patel et al., 1993], thus it is likely to be a major factor blocking the ability of NFS-60 cells to differentiate in response to cytokines such as G-CSF or Epo. The truncated 40–42 kD c-*myb* protein expressed in NFS-60 cells contains the same functional domains as the well-characterized v-*myb* and can also be expected to activate expression of *myb*-dependent genes such as c-*myc* [Cogswell et al., 1993]. It is interesting to note that MEL erythroleukemia cell lines overexpressing the c-*myc* gene also express elevated levels of Id-1 [Shoji et al., 1993]. These data taken together suggest a cascade of gene regulation in which c-*myb* then c-*myc* and finally Id-1 expression are sequentially activated during normal hematopoiesis. Minimally, the data imply that down-regulation of Id-1 is not a proximal event during the differentiation process. In the context of c-*myb*-overexpressing NFS-60 cells, arrested at a multipotent myeloid progenitor stage of differentiation, we show that Id-1 expression is still responsive to a broad selection of cytokines that can normally stimulate cells of that developmental age. Since Id-1 has been demonstrated to control expression/activity of important regulators of G_1 progression (e.g., the cyclin-dependent kinase inhibitor p21) [Prabhu et al., 1997], it is possible that Id-1 continues to be expressed until differentiation proceeds to the point at which maturing cells drop out of cycle, then it is extinguished.

Id-2 expression is stimulated by cytokines that activate granulocytic differentiation. Id-2 expression was detectable in logarithmically-growing cells and rose slightly (1.56-fold after adjustment for RNA loading) during cytokine withdrawal, when cell growth had arrested (Fig. 3). Although levels of Id-2 mRNA initially declined after cells were treated with stimulatory cytokines, cells cultured in either IL-6 or G-CSF for 24 h had elevated Id-2 levels (1.3- and 2.4-fold over that seen in arrested cells) while cells stimulated with other cytokines showed levels of Id-2 that declined to that less than that measured in the initial proliferating population. G-CSF drives maturing CFU-G progenitors along the neutrophil lineage [Nicola, 1989]; IL-6 has multiple targets during hematopoiesis, including stimulation of neutrophil progenitor proliferation and maturation as well as supporting function of mature neutrophils [Borregaard and Cowland, 1997]. Both of these factors are capable of stimulating neutrophil development directly, or through the induction of secondary cytokines.

TGFβ1 induces Id-2 expression and acts dominant to G-CSF. TGFβ1 is a bifunctional cytokine that inhibits growth and development of primitive progenitors while acting as a strong developmental stimulant for a population of relatively mature neutrophil precursors [Carlino et al., 1992; Hestdal et al., 1993]. To determine whether Id-1 or, especially, Id-2 might be a mediator of TGFβ1 differentiation control during hematopoiesis, we compared expression of these genes in cells treated with G-CSF (which strongly elevates Id-2 levels) and IL-3 (which does not affect Id-2 expression) or cells treated with TGFβ1. NFS-60 cells were starved of cytokines for 18 h; at this point, the cells had stopped proliferating. A portion of the arrested cells was induced to strong proliferation after treatment with IL-3 (1 U/ml) alone or in combination with TGFβ1 (100 ng/ml), or G-CSF alone (5 U/ml); when arrested cells were treated with G-CSF plus TGFβ1, TGFβ1 alone, or medium, they did not start to proliferate (Fig. 4A).

Id-1 was strongly induced by treatment with IL-3, alone or in combination with TGFβ1 (Fig. 4B); it was also induced, but to a lesser extent by G-CSF, G-CSF plus TGFβ1, or TGFβ1 alone (ranging from two-fold induction of Id-1 in cells treated with TGFβ1 alone to 11.3-fold in the cells treated with a combination of IL-3 and TGFβ1). Thus, Id-1 expression was most highly elevated in cells treated with any combinations including IL-3 and only slightly elevated after treatment with TGFβ1 alone (two-fold) or fresh medium (1.3-fold). The slight induction of Id-1 in the TGFβ1-fed population may reflect a feeding effect, nonspecific stimulation after addition of fresh culture medium.

The levels of Id-2 remained low in cells stimulated to proliferate by treatment with IL-3 or G-CSF (Fig. 4C). Cells treated with IL-3 plus TGFβ1 also grew briskly with low Id-2 levels

(Fig. 4C). Id-2 levels rebounded slightly by 24 h in the proliferating cell populations; this is likely due to the fact that these cultures were at or near confluence where cell proliferation stops and Id-2 is elevated normally.

In contrast to the effects of IL-3, cells stimulated with G-CSF plus TGFβ1 or TGFβ1 alone did not proliferate and showed strongly elevated Id-2 levels (7.16-fold over the initial proliferating population and 2.53-fold over the deprived cells that have arrested in growth and already elevated their Id-2 levels; Fig. 4C). Thus, the effect of TGFβ1 appears to be dominant to that of G-CSF with respect to Id-2 expression and cell growth. The opposite effect was observed for IL-3: NFS-60 cells stimulated by this cytokine and TGFβ1 proliferated strongly and did not elevate Id-2 levels. While IL-3-mediated proliferation of immature progenitor cells is generally inhibited by TGFβ1, maturing progenitors stimulated by IL-3 eventually become insensitive to these growth inhibitory effects [Lardon et al., 1994]. Thus TGFβ1 induction of Id-2 expression likely occurs at a more mature stage of development than the multipotent progenitor cell, analogous to the CFU-G granulocytic precursor.

These data taken together provide the basis for the following model. In multipotent myeloid progenitor cells, Id-1 responds similarly to all growth stimulatory signals, and is involved primarily in regulation of cell cycle progression. The regulation of Id-2 expression is more complex. Id-2 is induced as a selective response to cytokines that drive granulocytic development. During this process, cells lose their capacity to proliferate. Since Id-2 expression is inversely proportional to the degree of cell proliferation and it is strongly induced by conditions and cytokines that arrest cell growth, Id-2 functions as a terminal differentiation factor and mediates cell cycle arrest during granulopoiesis.

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