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Integrin-Associated Protein (IAP, Also Termed CD47) Is Involved in Stroma-Supported Erythropoiesis[1]

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Erythropoiesis is regulated by the hematopoietic microenvironment of the spleen, fetal liver, and bone marrow in mice. We previously showed that established stromal cells from these organs selectively support erythropoiesis *in vitro*. To identify the cell surface molecule(s) on the stromal cells involved in erythropoiesis, we raised monoclonal antibodies (MAbs) to MSS62 stromal cells derived from newborn spleen and obtained MAb-100.1, which partially inhibited the stroma-supported erythropoiesis *in vitro*. Using an expression cDNA library of MSS62 cells, we cloned a gene encoding the protein recognized by MAb100.1 and identified it as integrin-associated protein (IAP, also termed CD47), which may play a general role in integrin-mediated signal transduction. IAP/CD47 is expressed in the stromal cells of spleen, fetal liver, and bone marrow, and in a variety of hematopoietic cells including erythroblasts. Thus, IAP may be partly involved in the erythropoietic supporting ability of the stromal cells.

Key words: erythropoiesis, hematopoietic microenvironment, IAP/CD47, stromal cell.

Hematopoietic stem cells and their progenitor cells can proliferate and differentiate in the microenvironments of bone marrow, spleen, and fetal liver. Erythropoiesis is first observed in the yolk sac and then in the fetal liver (*1, 2*). In the adult mouse, the spleen is usually considered to be responsible for the stem cells being predominantly committed to erythroid development (*3*). Thus, it is thought that the stromal cells of mouse spleen may provide a microenvironment adequate for erythropoietic cells. We previously established stromal cell lines (MSS cells) from newborn spleen; as expected, these cells supported erythropoiesis *in vitro* (*4, 5*). Large erythroid colonies were formed from 13-day-old fetal liver erythroid progenitor cells in a semisolid medium containing erythropoietin (epo) in direct cell contact with stromal cell layers (*4, 6, 7*). Stromal cells with similar activity were established from fetal liver (*6, 7*) and bone marrow (*8*), indicating that erythropoiesis may be regulated by stromal cells of hematopoietic organs. By adding antibodies to the *in vitro* coculture system, we demonstrated that the production of SCF, a ligand for c-Kit, is essential (*7*), and that vascular cell adhesion molecule-1 (VCAM-1)-very late activation antigen-4 (VLA-4) interaction is necessary (*9*) for the stroma-supported erythropoiesis. However, these molecular interactions may not be sufficient for erythropoietic support of the stromal cells because of their partial inhibition by antibodies against these molecules. Thus, we searched for other functional molecules of the spleen stromal cells by raising monoclonal antibodies (MAbs) against surface molecules of MSS62, which inhibit the stroma-supported erythropoiesis *in vitro*. One such MAb, MAb100.1, was used for expression cloning of the gene encoding its antigen molecule. We found that a gene coding the protein recognized by MAb100.1 is identical to an integrin-associated protein (IAP, also termed CD47), an immunoglobulin family member.

MATERIALS AND METHODS

Screening of Monoclonal Antibodies against Stromal Cell Surface Molecules—MSS62, a mouse spleen stromal cell line established from the spleens of new born mice (*4*) was cultured in RITC80-7 (Kyokuto Pharmaceutical Industrial, Tokyo) supplemented with 2% FBS, 10 μg/ml bovine transferrin, 1 μg/ml insulin, and 10 ng/ml recombinant epidermal growth factor (EGF, generously supplied by Wakunaga Seiyaku, Tokyo). Cells were passaged by trypsin treatment at one week intervals. MSS62 cells in monolayer culture were washed with PBS (Dulbecco's phosphate-buffered saline) and recovered with a cell scraper. Intact cells (1×10^7 cells/injection) were injected into 6-week-old Wistar rats for immunization three times at 2-week intervals. After the last booster, immune splenocytes were fused with mouse myeloma (NS-1) cells with 50% polyethylene glycol (PEG4000). After HAT selection, the supernatants of growing hybridomas were subjected to

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primary screening by means of an enzyme-linked immunosorbent assay (ELISA) using methanol-fixed MSS62, and BALB/3T3 fibroblast cells (cultured with 10% FBS-supplemented DMEM) as a negative control, followed by cloning. After inhibition assaying of large erythroid colony formation, 5 hybridomas were obtained from about 300 ELISA-positive hybridomas. One of these hybridomas (100.1) was selected for expression cloning. To purify the 100.1 monoclonal antibody in the culture supernatant, the monoclonal antibody was precipitated with ammonium sulfate, followed by affinity chromatography on Protein G- Sepharose (Pharmacia, Uppsala, Sweden).

Inhibitory Activity of MAbs with the Large Erythroid Colony Formation Assay—The inhibitory activity of MAbs was assayed as to large erythroid colony formation (*4*). For large erythroid colony formation, 2×10^4 MSS62 cells were seeded to form a monolayer in a 24-well plate one day before cocultivation. Erythroid progenitors prepared from the livers of C57BL/6J mice at 13-day gestation were suspended in Iscove's modified Dulbecco's modification of Eagle's medium (IMDM; GIBCO, Grand Island, NY) with 30% heat-inactivated FBS, 0.4% methylcellulose, 1% BSA, 100 μM 2-mercaptoethanol, and 0.1 U/ml Epo (recombinant erythropoietin; generously supplied by Kirin, Tokyo), and then added to the MSS62 stromal cell layers. To this coculture, 5 to 10 μg/ml of MAbs was added, followed by culturing for 4 days. Then, the cultures were directly stained with a 1/10 volume of a benzidine solution (12.9% acetic acid, 0.43% benzidine, and 4.3% hydrogen peroxide) to detect the large erythroid colonies consisting of hemoglobin-producing cells.

Construction of a Directional cDNA Library—Poly(A)$^+$ RNA was isolated from MSS62 cells with a mRNA isolation kit (FastTrack 2.0 Kit; Invitrogen, San Diego, CA). First strand cDNA was synthesized from 5 μg of poly(A)$^+$ RNA using the *Not*I/oligo-(dT)$_{18}$ primer (Directional Cloning Tool Box; Pharmacia) to obtain directionally cloned cDNA. Following double-strand cDNA synthesis (TimeSaver cDNA Synthesis Kit; Pharmacia), an *Eco*RI adaptor was attached and digested with *Not*I. The cDNA larger than 1.0 kb was fractionated by agarose gel electrophoresis and the size-selected cDNA was inserted into the *Eco*RI/*Not*I site of the pME18S vector, a derivative vector of pCEV4 (*10*). Electroporation-competent *Escherichia coli* cells (ElectroMAX DH10B cells; GIBCO-BRL) were used for transformation and approximately 5.7×10^6 independent clones were obtained.

Screening of the cDNA Library as to Expression—The cDNA library was introduced into COS7 cells by the spheroplast fusion method (*11*). For the first round of screening, forty 60 mm dishes (Falcon PRIMARIA 3802; Becton Dickinson, San Jose, CA) of subconfluent COS7 cells were transfected. After 3 days, cells were detached by incubation in PBS containing 1 mM EDTA at 37°C for 30 min. The cells were collected by centrifugation and resuspended in cold PBS containing 5% FBS at 5×10^6 cells/ml, and then MAb100.1 was added at 10 μg/ml. In every round of screening, $5.0\text{-}5.6\times10^7$ cells were used. After 30 min incubation on ice, the cells were washed with PBS three times and then resuspended in cold PBS containing 5% FBS at 5×10^6 cells/ml. Anti-rat IgG-coated magnetic beads (Dynabeads; Dynal AS, Oslo, Norway) were added to the cell suspension in the proportion of one cell to 4 beads. After 30 min incubation on ice, the cell suspension was stood in a magnetic holder (MPC-1; Dynal) for 3 min. The nonadsorbed cells were removed with a pipette and the adsorbed cells were resuspended in PBS. This adsorption was repeated until no cells were observed on washing with PBS. Finally, 2.5 ml of the cell lysis solution [0.6% sodium dodecyl sulfate (SDS), 10 mM EDTA] was added per tube, followed by incubation for 20 min at room temperature. The beads were removed by standing in the magnetic holder and the cell lysate was transferred to microtubes. Plasmid DNA was recovered from the cell lysate (*12*) and used to transform *E. coli* by electroporation. The *E. coli* transformants were amplified and used for the next round of screening.

DNA Sequencing—The cDNA inserts were subcloned into the *Eco*RI/*Not*I site of the pBluescript II KS(+) vector (STRATAGENE, La Jolla, CA), and sequenced with an automatic sequencer (ALF DNA Sequencer; Pharmacia). The obtained sequences were searched for in the Genbank and EMBL nucleotide databases.

mRNA Detection by RT-PCR—To detect the spliced form of IAP mRNA, RNAs were extracted from C57BL/6 mouse tissues and cell lines by the acid phenol procedure using ISOGEN (Wako Pure Chemicals, Tokyo) according to the manufacture's protocol. The RT-PCR method was performed to detect spliced forms of IAP mRNA from various tissues, spleen stromal cells (MSS62), bone marrow stromal cells (TBR59 and TBR31-1) (*13*), and lymphoid cells (B31-1). Total RNAs were reverse-transcribed with MMLV-reverse transcriptase (GIBCOBRL, Grand Island, NY), and the cDNAs were amplified with *Taq* DNA polymerase (TaKaRa, Tokyo) and two primers, (5′-GCGA-AGTGACAGAGTTATCC-3′) and (3′-TGGCTCACATGC-CATGATGC-5′), for 30 cycles consisting of successive incubations at 94°C (30 s), 58°C (60 s), and 72°C (60 s). The amplified dsDNAs were analyzed by agarose gel electrophoresis.

Flow Cytometry—MSS62 and transfected COS7 cells were detached by incubation in PBS containing 1 mM EDTA at 37°C for 30 min, then suspended in the cold washing buffer (PBS containing 0.2% BSA and 1 mM EDTA) at 1×10^6 cells/ml. MAb100.1 was added to the cell suspension at 10 μg/ml, followed by incubation for 30 min on ice. The cells were washed three times with the washing buffer and then resuspended in the washing buffer containing fluorescein isothiocyanate (FITC) conjugated anti-rat IgG (ORGANON TEKNIKA, Durham, NC). After 30 min incubation on ice, the cells were washed three times with the washing buffer and then analyzed with a FACStar^{PLUS} (Becton Dickinson).

RESULTS

Screening of Monoclonal Antibodies Which Inhibit Stroma-Supported Erythropoiesis—To raise MAbs against surface molecules of the mouse spleen stromal cell line, MSS62, intact MSS62 cells were injected into rats. After three successive injections, splenic cells from the immunized rats were hybridized with mouse myeloma (NS-1) cells. Approximately 2,000 antibody-producing hybrid cell clones were obtained and approximately 300 clones were selected as being MSS62-positive, BALB 3T3-cell-negative clones on ELISA assaying. Then, their inhibitory

activity toward the stroma-supported erythropoiesis *in vitro* was examined by means of large erythroid colony formation assaying of the epo-responsive fetal liver progenitor cells in the presence of epo on the MSS62 stromal cell layers (*4, 6*), and we found that MAb100.1 significantly inhibited the stroma-supported erythropoiesis (25-35%) (Fig. 1). FACS analysis involving MAb100.1 indicated that the protein recognized by MAb100.1 was localized on the surface of MSS62.

Expression Cloning with MAb100.1—Anticipating that MAb100.1 may recognize a candidate functional molecule of the stromal cells for erythropoietic supporting activity, we tried to isolate the gene encoding the protein recognized by MAb100.1 by expression cloning. For expression cloning using MAbs, we constructed a cDNA library using Poly(A)$^+$ RNA isolated from MSS62 stromal cells. The cDNA library was introduced into COS7 cells by the spheroplast fusion method followed by culturing for 3 days to transiently express proteins. The transfected cells expressing surface molecules recognized by MAb100.1 were collected by the addition of this antibody, followed by the addition of anti-rat IgG conjugated magnetic beads and adsorption to a magnet. Plasmid DNAs were recovered from the adsorbed cells and transfected into *E. coli* for the next round of screening. After each round of screening, a portion of the fused COS7 cells was used for analysis of expression of its antigen by FACS, and the amplified plasmid DNAs were analyzed by restriction enzyme digestion to monitor the degree of enrichment. These procedures were repeated to concentrate the cDNA clone encoding the surface molecule recognized by MAb100.1. Whereas maximum staining was observed in MSS62, COS7 cells did not show any staining (Fig. 2A). In the third round of enrichment, approximately 2% of the cells were MAb100.1-positive and among 24 independently isolated plasmid DNAs, 11 had the same 1.3 kb cDNA insert. In the fourth round, approximately 11% of the COS7 cells were MAb100.1-positive (Fig. 2B). Restriction enzyme analysis of the amplified plasmid DNAs showed that the band of 1.3 kb became clearer with increasing positive cells on FACS analysis (Fig. 2C). Eight of 12 clones had the same 1.3 kb cDNA insert (Fig. 2D), and all the 1.3 kb cDNAs had at least a single recognition site for *Nco*I and *Pvu*II (data not shown). Thus, we concluded that these 1.3 kb cDNA inserts encoded the MAb100.1 antigen.

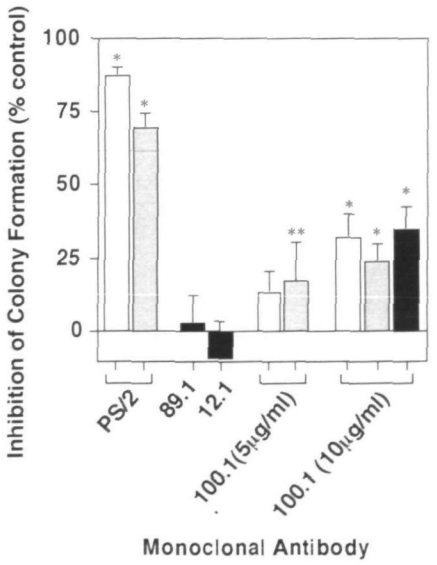

Fig. 1. **Inhibition of large erythroid colony formation by MAb100.1.** For large erythroid colony formation, 1×10^4 fetal liver cells were cultured on MSS62 cells in 24-well plates. After 4 days, benzidine-positive colonies were scored. Open column: experiment 1, dotted column: experiment 2, and filled column: experiment 3. PS/2: Rat monoclonal antibody recognizing mouse $\alpha 4$ subunit of integrin VLA-4. MAb89.1 and 12.1 were ELISA-positive clones established at the same time with MAb100.1, and these three MAbs are IgG2b. All MAbs were added at 10 μg/ml, except for MAb100.1, 5 μg/ml. The numerical values are % of the control for 3 (Experiments 2 and 3) or 4 wells (Experiment 1), and the vertical bars represent standard deviation ($^*p < 0.005$, $^{**}p < 0.05$).

Identification of the MAb100.1-Antigen as IAP—The sequencing of 4 independent clones showed that they contained an ORF identical to IAP. On comparison of the nucleotide sequences of different cDNA clones with mouse, rat, and human cDNAs, we found that a stretch (21 amino acid residues) of the mouse IAP sequence which is absent in the human and rat sequences was also missing in 3 of our clones, while the other clone was completely identical to the published mouse IAP cDNA within the ORF (Fig. 3). Thus, the differences between the published human, rat, and mouse sequences may be due to differentially spliced forms, and IAP mRNAs expressed in MSS62 cells contain both spliced forms.

Some alternatively spliced forms of IAP mRNA have been reported in hematopoietic cells of mouse and human (*14-16*), but most of them are located in the intracytoplasmic carboxy-terminal and expression of different spliced forms varies among hematopoietic cell lines (*15, 17-19*). Thus, the expression of a new spliced form with a 21-amino acid deletion in the external region found in MSS62 stromal cells was measured in a hematopoietic cell line, and stromal cells from different origins and different tissues by RT-PCR. All cell lines and tissues showed both spliced forms at equal frequency (Fig. 4).

DISCUSSION

Although erythropoiesis is regulated by erythropoietin *in vitro* or *in vivo*, the rapid expansion of erythroid progenitor cells is also dependent on the interaction with stromal cells of erythropoietic organs, as demonstrated by the *in vitro* culture of these progenitor cells on stromal cells established from erythropoietic organs of mice (*4-6*). Using this stroma-supported erythropoiesis *in vitro*, it is possible to identify the functional molecules involved in the erythropoietic organs. We examined the roles of adhesion molecules in erythropoiesis by blocking antibodies in this stroma-supported erythropoiesis (*9*). The development of erythroid cells on stromal cells was inhibited by an anti-VLA-4 integrin antibody, but not by an anti-VLA-5 antibody, although the erythroid cells expressed both VLA-4 and VLA-5. High levels of expression of VCAM-1, a ligand for VLA-4, were detected in the stromal cells, and the adhesion and development of the erythroid progenitor cells were inhibited by the blocking antibody against VCAM-1, although the inhibition was partial. VLA-5 could mediate the adhesion of the erythroid progenitor cells to the stromal cells, but the adhesion itself may not be sufficient for the stroma-supported erythropoiesis. Upon transfection of the v-*src* oncogene into MSS62 cells, the transfectants with

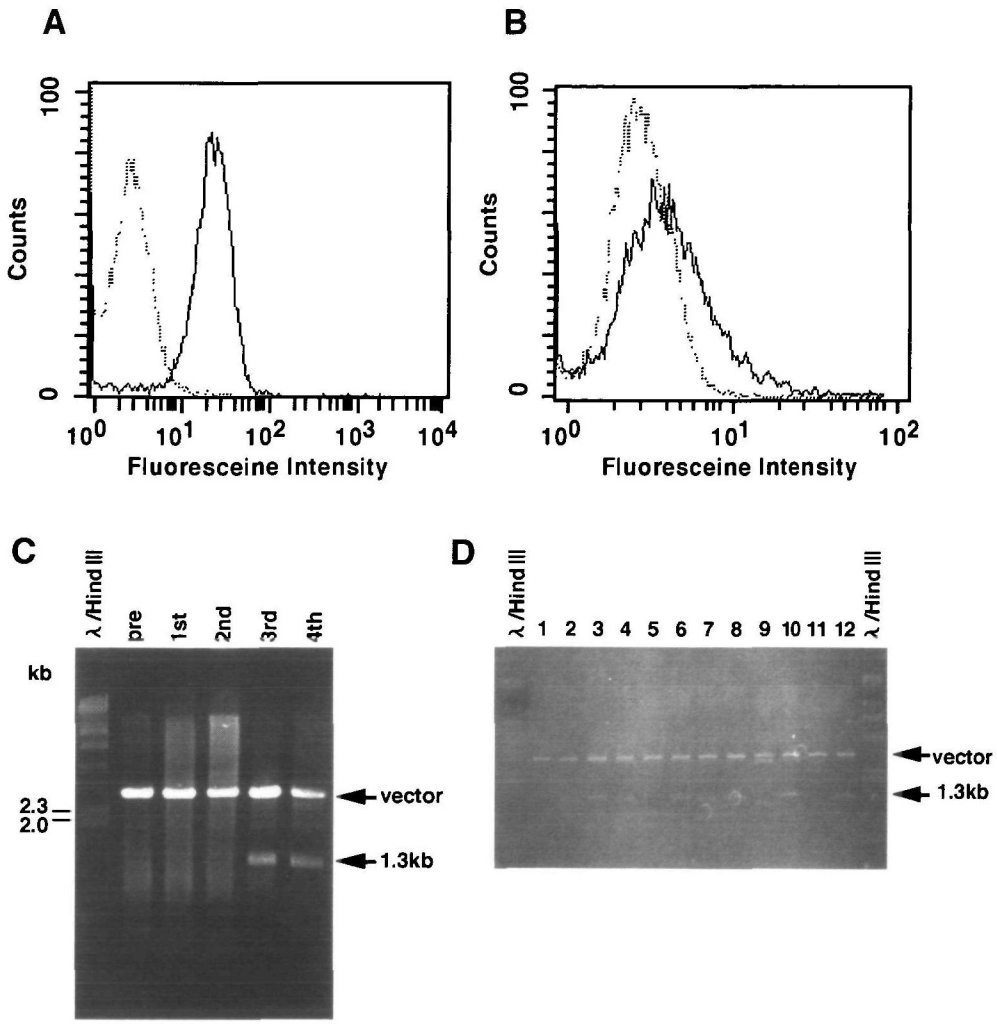

Fig. 2. **(A) FACS profiles of MSS62 cells (solid line) and COS7 cells (dotted line) stained with MAb100.1. (B) FACS analysis of COS7 cells with the cDNA library introduced on the fourth round of enrichment.** Approximately 11% of the cells reacted with MAb100.1. The dotted line is the profile for the cells treated with FITC-conjugated anti-rat IgG alone as a background. **(C) The amplified plasmid DNAs at each round of enrichment were digested with *Eco*RI and *Not*I, and then analyzed by agarose gel electrophoresis.** The band of 1.3 kb became clearer with each round of enrichment. **(D) After the fourth round, 8 of 12 clones had the same 1.3 kb insert.**

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mouse IAP   101 DKRDAM--VGNYTCEVTELSREGKTVIELKNRTAFNTDQGSACSYEEEKG  148
clone 2     101 DKRDAM--VGNYTCEVTELSREGKTVIELKNRTAFNTDQGSACSYEEEKG  148
clone 4     101 DKRDAM--VGNYTCEVTELSREGKTVIELKNRT----------------  131
rat IAP     101 DTHEAV--VGNYTCEVTELSREGKTVIELKNRP----------------  131
human IAP   101 DKSDAVSHTGNYTCEVTELTREGETIIELKYRV----------------  133

mouse IAP   149 GCKLVSWFSPNEKILIVIFPILAILLFWGKFGILTLKYKSSHTNKRIILL  198
clone 2     149 GCKLVSWFSPNEKILIVIFPILAILLFWGKFGILTLKYKSSHTNKRIILL  198
clone 4     132 ----VSWFSPNEKILIVIFPILAILLFWGKFGILTLKYKSSHTNKRIILL  177
rat IAP     132 ----VSWFSTNEKILIVIFPILAILLFWGKFGILTLKYKSSHTNKRIILL  177
human IAP   134 ----VSWFSPNENILIVIFPIFAILLFWGQFGIKTLKYRSGGMDEKTIAL  179
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Fig. 3. **Different spliced forms of IAP found in MSS62 cells.** The amino acid sequences deduced from cDNAs obtained in this study (clones 2 and 4) were aligned with those of the mouse (*14*), rat (*33*), and human IAPs (*14*). The sequences were numbered starting with the initiator methionine, the 98 residues from 101 with the gaps indicated (-) being shown. In clone 4, there was a 21-amino acid deletion similar to that in the rat and human IAPs.

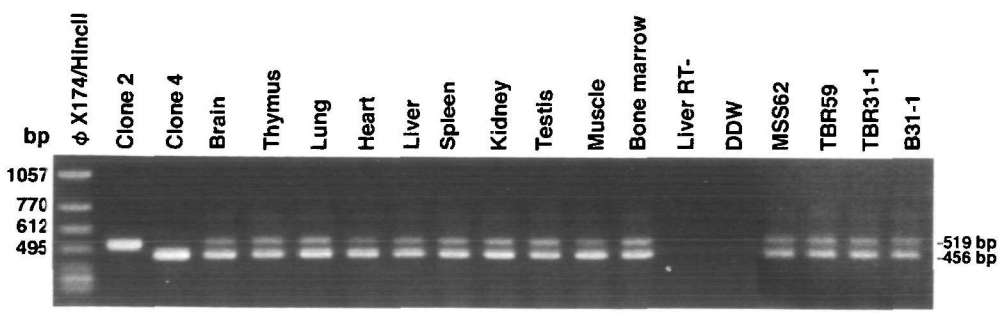

Fig. 4. **Different spliced forms of IAP/CD47 mRNA expressed in various tissues and cell lines.** Total RNAs from various tissues and cell lines (MSS62, TBR59, and TBR31-1 are stromal cell lines. B31-1 is a hematopoietic cell line) were subjected to RT-PCR with the primers that detected the spliced forms of IAP/CD47 mRNA. Liver RT-: Liver RNA without reverse transcriptase reaction. DDW: Water was applied instead of cDNA. Two spliced forms were detected in all samples.

high v-Src activity exhibited low erythropoietic stimulatory activity (*20*). A decrease in the expression of cell-surface VCAM-1 was accompanied by high v-Src activity, suggesting that v-Src interferes with the erythropoietic stimulatory activity of the stromal cells through repression of adhesion molecules.

In addition to the above mentioned known molecules, we searched for others by raising MAbs against the surface of the stromal cells and obtained MAb100.1, which partially inhibited large erythroid colony formation. Isolation of the gene recognized by this MAb indicated that its antigenic protein is IAP/CD47, which may be a functional molecule for the stroma-supported erythropoiesis.

IAP was first identified by Brown and co-workers (*17*) from its association with $\alpha V\beta 3$ integrin. It is a 50-kDa membrane protein with an amino-terminal immunoglobulin domain and a carboxyl-terminal 5 membrane-spanning region, and is expressed on a variety of cell types including hematopoietic cells and stromal cells (*15, 17-19*).

Some alternatively spliced forms of IAP mRNA have been reported in mouse and human (*14-16*). Whereas most of them are located in the intracytoplasmic carboxy-terminal, we found a new spliced form of mouse IAP with a 21-amino acid deletion in the external region. Lindberg *et al.* (*21*) have reported that the Ig variable domain (IgV) of IAP is necessary for the adhesion of cells to vitronectin-coated beads. Although in our new spliced form the IgV region was not deleted, it may affect the structure of IAP and thus may affect its function. We showed that both spliced forms of IAP mRNA are expressed in an almost equal ratio in a variety of tissues, including hematopoietic cells and stromal cells.

IAP appears to be involved in signal transduction by αV and perhaps other integrins since MAbs directed to IAP block integrin-stimulated phagocytosis (*17, 22*), an entactin-stimulated oxidant burst in neutrophils and monocytes (*23, 24*), and the inward calcium current in endothelial cells (*25*) adhering to fibronectin. IAP was also shown to be a receptor for the C-terminal domain of thrombospondin (*26*), which belong to a family of proteins implicated in the regulation of the motility, proliferation, and differentiation of many cell types. Lindberg *et al.* (*27*) recently reported that IAP-deficient mice showed a defect in host defense, probably secondary to both delayed polymorphonuclear leucocyte (PMN) migration to the site of infection and defective activation at that site. Because IAP acts as both a component of $\beta 3$ integrin signaling and a receptor for the ECM protein, thrombospondin, it would be ideally suited for integrating signals from several matrix proteins to inform the PMNs that it is in a tissue, rather than in the blood stream.

IAP was recently shown to be identical to the 1D8 antigen and CD47, which is reduced on Rhnull erythrocytes (*28-31*). Lindberg *et al.* (*14*) showed that some MAbs are capable of inhibiting the vitronectin binding of erythroleukemia cells. These results suggested a role of IAP in erythropoiesis or the erythrocyte function. Although we have demonstrated MAb 100.1 recognizes IAP on the stromal cells, it is likely that IAP may be a functional molecule on erythroid progenitor cells and that this MAb may inhibit the interaction of erythroid cells with stromal cells. Ticchioni *et al.* (*32*) recently reported that IAP cross-linking with a specific MAb transduces costimulatory signals within highly purified CD3-activated T lymphocytes. Whereas there is no functional association between IAP and integrins on T lymphocytes, IAP cross-linking induces the phosphorylation of $p56^{lck}$ protein tyrosine kinase, and IAP is associated with $p56^{lck}$ in PMA-activated, but not resting T cells. They suggested that IAP may be involved directly *via* a specific ligand in cell-matrix or cell-cell interactions in both the maturation and activation of T cells. A similar mechanism may be at work on stromal cells to support rapid expansion of the erythroid progenitors.

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