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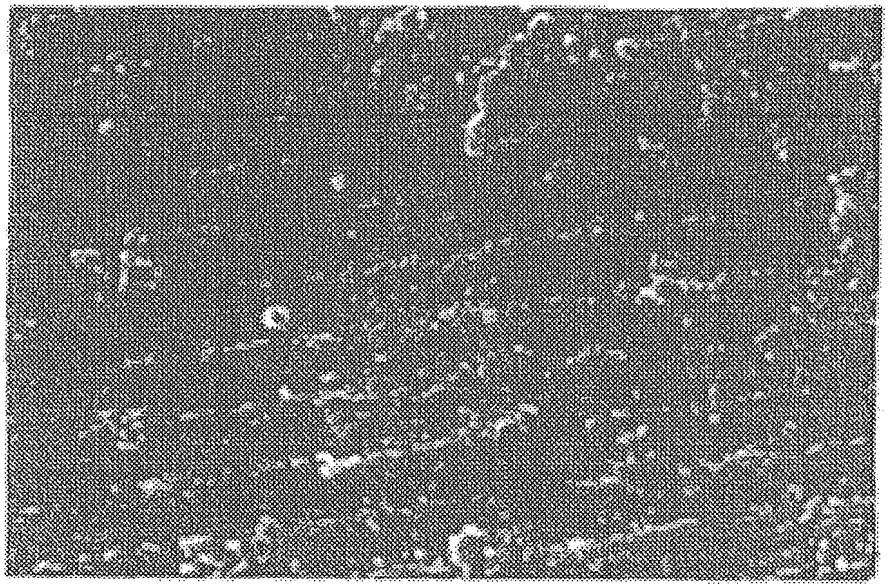
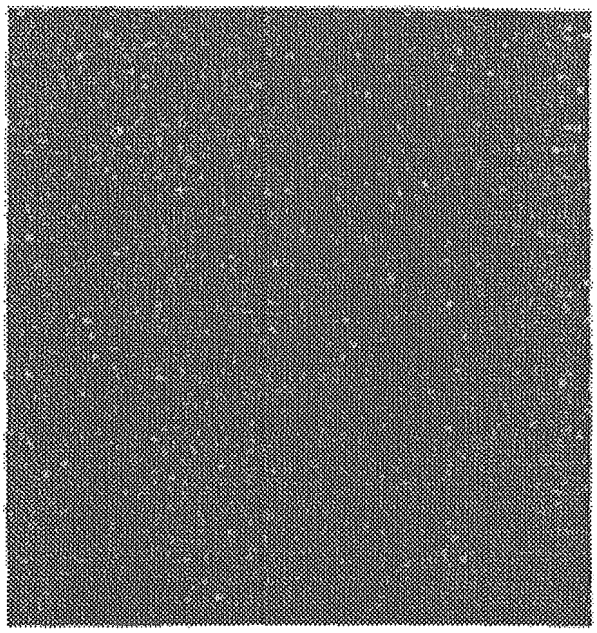
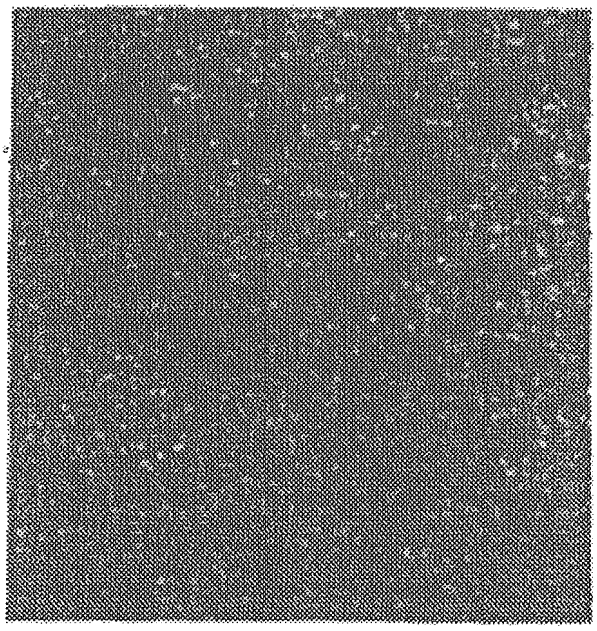


Figure 6



Precatenated



Catenated

METHODS FOR ISOLATION OF OSTEOCLAST PRECURSOR CELLS AND INDUCING THEIR DIFFERENTIATION INTO OSTEOCLASTS

TECHNICAL FIELD

The present invention relates to a method for the differentiation of osteoclast precursor cells (preosteoclasts) into osteoclasts, which comprises culturing the preosteoclasts in the absence of accesory cells; a method for isolating preosleoclasts; a method for screening agents for metabolic bone diseases, which comprises using the preosteoclasts or the osteoclasts; and agents for metabolic bone diseases, which is obtainable by the screening method.

BACKGROUND ART

Bone tissues of mammal repeat generation and resorption of bone. The tissue operates as a central point of calcium metabolism to keep the balance between bone resorption and generation in the growth period and even after the period of maturity. Bone resorption and resorption are well balanced by crosstalk between osteoclasts and osteoblasts. However, unbalance between bone resorption and generation lead metabolic bone diseases, including osteoporosis, rheumatoid arthritis, osteoarthritis, decrease of bone quantity due to diabetes, many types of hormone abnormalities, nutritional disorder, osteopetrosis and osteomalacia. Cellular pathogenesis of the most of above disorders remain to be elucidated. To resolve the issue and discover therapeutic agents for the metabolic bone diseases, methods for isolation and characterization of osteoclasts and osteoblasts have been required.

It has been studied well for isolation of osteoclasts of mice or rats. Recentry, Fujisawa et al. reported that human osteoclasts were obtained from patients with rheumatoid arthritis (Annals of Rheumatic Diseases. 55;816–822, (1996)). In their study, human osteoclasts were obtained in the presence of a mouse osteoblasts-like cell line, since it has been believed that the presence of accessory cells, such as osteoblasts or bone marrow stromal cells, were indispensable for differentiation of preosteoclasts in vitro. The accessory cells have been believed to play important role for osteoclastogenesisi by close contact with preosteoclasts.

On the other hand, JP 7-194373 describes a method for the differentiation of bone mallow cells into osteoblasts, osteoclasts or chondrocytes in a medium without bone mallow stromal cells or osteoblasts. But, they did not succeed in the isolation of osteoclasts itself. Under the circumstances above, in order to elucidate the mechanism of differentiation of preosteoclasts into osteoclasts, it was required to establish a method for isolation of preosteoclasts, and that for inducing the differentiation into osteoclasts, which needs few factors for the differentiation, and particularly, especially in the absence of accessory cells.

DISCLOSURE OF INVENTION

As a result of intensive studies on the induction of osteoclastgenesis, the present inventors have accomplished inventions on a method for differentiating preosteoclasts into osteoclasts which comprises culturing the osteoclast precursor cells in the absence of accessory cell, a method for isolating preosteoclasts, a preosteoclast which is isolated by the isolating method, a method for differentiating the preosteoclasts isolated by the isolating method into osteoclasts, a osteoclast which is obtainable by the differentiating method, a method for screening agents for metabolic bone diseases and an agent for metabolic bone diseases which is obtainable by the screening method.

The invention relates to:

① A method for differentiating preosteoclasts into osteoclasts, which comprises culturing the preosteoclasts in the absence of accessory cells;

② The method as described in ①, which uses a culture medium containing IL-3, IL-7, GM-CSF, eotaxin, eotaxin-2, eotaxin-3 or a mixture of two or more of them;

③ The method as described in ① or ②, which uses a culture medium containing a culture supernatant of mitogen-stimulated peripheral blood mononuclear cells;

④ The method as described in ③, wherein the culture supernatant of mitogen-stimulated peripheral blood mononuclear cells is a culture supernatant of phytohemagglutinin-stimulated human peripheral blood mononuclear cells;

⑤ A method for isolating preosteoclasts, which comprises culturing peripheral blood or joint fluid in the absence of cytokine for 1 to 3 weeks;

⑥ The method as described in ⑤, in which the preosteoslasts are isolated by adding peripheral blood or joint fluid to essential medium for mammalian cells in the absence of cytokine and culturing them at 35–37° C. in 5–7% CO_2-containing air for 1–3 weeks to perish cells except preosteoclasts;

⑦ An preosteoclast, which is obtainable by the method described in ⑤ or ⑥;

⑧ A method for differentiating preosteoclasts obtained by the method as described in ⑤ or ⑥ into osteoclasts, which comprises culturing the preosteoclasts in the absence of accessory cells;

⑨ The method as described in ⑧, which uses a culture medium containing IL-3, IL-7, GM-CSF, eotaxin, eotaxin-2, eotaxin-3 or a mixture of two or more of them;

⑩ The method as described in ⑧ or ⑨, which uses a culture medium containing a culture supernatant of mitogen-stimulated peripheral blood mononuclear cells;

⑪ The method as described in ⑩, wherein the culture supernatant of mitogen-stimulated peripheral blood mononuclear cells is a culture supernatant of phytohemagglutinin-stimulated human peripheral blood mononuclear cells;

⑫ An osteoclast, which is obtainable by the method as described in any one of ① to ④ and ⑧ to ⑪;

⑬ A method for screening agents for metabolic bone diseases, which comprises using the preosteoclasts isolated by the method as described in ⑤ or ⑥;

⑭ A method for screening agents for metabolic bone diseases, which comprises using the preosteoclasts as described in ⑦;

⑮ A method for screening agents for metabolic bone diseases, which comprises using the osteoclasts obtained by the method as described in any one of ① to ④ and ⑧ to ⑪.

⑯ A method for screening agents for metabolic bone diseases, which comprises using the osteoclasts as described in ⑫;

⑰ An agent for metabolic bone diseases, which is obtainable by the method as described in any one of ⑬ to ⑯.

One of the invention relates to a method for differentiating preosteoclasts into osteoclasts, which comprises culturing the preosteoclasts in the absence of accessory cell. In this case, "accessory cell" means mesenchymal cell which can induce the differentiation of preosteoclasts by producing adhesive factors and soluble factors. As a "accessory cell", bone mallow stromal cells, osteoblasts (osteoblast like cells), fibroblasts and tumor cells are given for examples. "Osteoclast precursor cells" or "Preosteoclasts" means cells which do not substantially contain any admixture cell. Concretely, it means human preosteoclasts. "Osteoclast precursor cells" or "Preosteoclasts" also means hematopoietic stem cell-derived cells which have an ability of differentiating into osteoclasts under an appropriate culture condition. "Osteoclasts" means cells which do not substantially contain any admixture cells. Concretely, it means human osteoclasts. "Osteoclasts" also means cells which are multinucleate (N>3), positive for tartrate-resistant acid phosphatase, and have an ability of bone resorption.

Further, this invention relates to a method for differentiating preosteoclasts into osteoclasts, which uses a culture medium containing IL-3, IL-7, GM-CSF, eotaxin (Kitaura, M. et al., J. Biol. Chem., 271, 13, 7725–7730, 1996), eotaxin-2 (Forssmann, U. et al., J. Exp. Med., 185, 12, 2171–2176, 1997), eotaxin-3 (human CC type chemokine. The nucleic acid sequence is shown at SEQ ID No.:1, and the amino acid sequence is shown at SEQ ID No.:2) or a mixture of two or more of them. IL-3, IL-7, GM-CSF, eotaxin, eotaxin-2 and eotaxin-3, each may be natural type or recombinant type. As the culture medium which contains IL-3, IL-7, GM-CSF, eotaxin, eotaxin-2, eotaxin-3 or a mixture of two or more of them, a culture supernatant of mitogen-stimulated peripheral blood mononuclear cells can be used. As the culture supernatant of mitogen-stimulated peripheral blood mononuclear cells, a culture supernatant of phytohemagglutinin-stimulated human peripheral blood mononuclear cells can be used.

The invention also relates to a method for isolating preosteoclasts by culturing peripheral blood or joint fluid in the absence of cytokine for 1 to 3 weeks. In detail, the invention relates to a method for isolating preosteoslasts by adding peripheral blood or joint fluid to essential medium for mammalian cells in the absence of cytokine, and culturing them at 35–37° C., in 5–7% CO_2-containing air for 1–3 weeks to perish cells except preosteoclasts. Further, the invention relates to preosteoclasts which are obtainable by the method.

"Peripheral blood" means mammalian peripheral blood, concretely, human peripheral blood. As the peripheral blood, peripheral blood of healthy donors subjects can be used.

As "joint fluid", not only joint fluid of healthy donors but also joint fluid of rheumatoid arthritis (RA) subjects can be used.

"Essential medium for Mammalian cells" means an isotonic buffer solution which contains inorganic salts, essential amino acids or its derivatives, and vitamin and its derivatives, which are available for cells to survive. As the "essential medium for mammalian cells", Dulbecco's Modified Eagle Medium(DMEM), RPMI1640 and AIM-V are given for examples.

Further, the invention relate to a method for differentiating preosteoclasts into osteoclasts, which comprises culturing in the absence of accessory cell the preosteoclasts which are isolated by culturing the peripheral blood or joint fluid as described previously in the absence of cytokine for 1 to 3 weeks. Concretely, the invention relates to a method for differentiating preosteoclasts into osteoclasts, which uses a culture medium containing IL-3, IL-7, GM-CSF, eotaxin, eotaxin-2, eotaxin-3 or a mixture of two or more of them. As IL-3, IL-7, GM-CSF, eotaxin, eotaxin-2 or eotaxin-3, each may be natural type or recombinant type. As a culture medium which contains IL-3, IL-7, GM-CSF, eotaxin, eotaxin-2, eotaxin-3 or a mixture consisted by a combination of two or more of them, a culture supernatant of mitogen-stimulated peripheral blood mononuclear cells is available. As a culture supernatant of mitogen stimulated peripheral blood mononuclear cells, a culture supernatant of phytohemagglutinin stimulated human peripheral blood mononuclear cells is available.

As another embodiment, the invention relates to a method for screening agents for metabolic bone diseases, which comprises using the preosteoclasts or the osteoclasts of this invention, and to an agent for metabolic bone diseases which is obtainable by the screening method. As the "metabolic bone disease", osteoporosis, rheumatoid arthritis, decrease of bone quantity due to diabetes, osteomalacia, and osteopetrosis are given for example.

As "a method for screening agents for metabolic bone diseases", (1) a method for measuring inhibitory activities on the differentiation of preosteoclasts into osteoclasts, and (2) a method for measuring inhibitory activities on the bone resorption by osteoclasts are given for example. If a candidate compound for the therapeutic agent shows a differentiation inhibitory activity against the preosteoclasts of this invention, the compound is promising as an antirheumatic drug. Futher, if a candidate compound for the therapeutic agent shows a bone resorption inhibitory activity against the osteoclasts of this invention, it is suggested that the compound is useful for treating osteoporosis caused by excessive bone resorption, decrease of bone mass due to diabetes, osteomalacia or the like. Thus, by using the preosteoclasts or the osteoclasts of this invention, the screening for bone metabolic diseases agents can easily be performed in vitro.

BRIEF DESCRIPTION OF DRAWINGS

FIG. **1** is photomicrographs which show that the preosteoclasts and the osteoclasts stained by May-Giemsa solution.

FIG. **2** is photomicrographs which show that the TRAP activities in the preosteoclasts and the osteoclasts.

FIG. **3** is photomicrographs which show the resorption activities of the preosteoclasts and the otsteoclasts, which was tested by using dentine slices.

FIG. **4** is a photomicrograph which shows resorption pits on dentine slice formed by the osteoclasts.

FIG. **6** is photomicrographs which show the resorption activities of the preosteoclasts and the otsteoclasts, which was tested by using a hydroxyappatite sintering quartz disc.

BEST MODE FOR CARRYING OUT THE INVENTION

The human osteoclasts of the invention can be obtained by the following methods.

Isolation of Preosteoclasts

Isolation of osteoclast precursor cells comprises (1) separation step of cellular fraction and (2) isolation step of preosteoclasts.

(1) Separation of Cellular Fraction

Preotsteolasts can be obtained from peripheral blood of healthy donors or joint fluid of rheumatoid arthritis subjects. The volume of bodily fluids necessary for isolating the cells

is 50 ml to 200 ml for peripheral blood of normal subjects, or 1 ml to several tens ml for joint fluid of rheumatoid arthritis subjects. In the case of using peripheral blood of healthy donors, the blood is collected in the presence of heparin or an alternative anticoagulant. Peripheral blood mononuclear cells (PBMC) are obtained from the blood by excluding of erythrocyte using a specific gravity centrifugation or ammonium chloride so on.

On the other hand, in the case of using joint fluid of rheumatoid arthritis subjects, the cellular fraction can be obtained by the centrifugation of the joint fluid, if necessary by adding essential medium for mammalian cells, such as RPMI1640, thereto.

(2) Isolation of Preosteoclasts

The obtained cellular fraction is cultured in essential medium for mammalian cells, such as DMEM, at 35–37° C., preferably, 37° C., for several weeks, preferably, 1–3 weeks, in 5–7% CO_2-containing air. This culture make cells except preosteoclasts perish, and the preosteoclasts can be isolated.

Isolation of Osteoclasts

Isolation of osteoclasts comprises (1) preparation step of culture medium and (2) differentiation-inducing step of preosteoclasts into osteoclasts.

(1) Preparation of Culture Medium

The medium is prepared by an addition of cytokines to essential medium for mammalian cells, such as AIM-V to induce the differentiation. Preferably, antibiotics are added to the medium for preventing the contamination of bacteria. According to the invention, IL-3, IL-7, GM-CSF, eotaxin, eotaxin-2, eotaxin-3 are given for example as the cytokine which have an activity to induce the differentiation of the preosteoclasts. These cytokines may be added as alone or mixture thereof.

(2) Differentiation Induction of Preosteoclasts into Osteoclasts

The obtained preosteoclsats are cultured at 35–37° C., for 48–96 hours in the medium described above. The preosteoclasts are differentiated into osteoclasts by the stimulation of the cytokines described above. Thus, the osteoclasts can be isolated.

Screening of Therapeutic Agents for Metabolic Bone Diseases

As a method for screening therapeutic agents for metabolic bone diseases, (1) a method for measuring inhibitory activities on the differentiation of preosteoclasts into osteoclasts, and (2) a method for measuring inhibitory activities on bone resorption by the osteoclasts are given for example.

(1) Measuring Method of Differentiation Inhibitory Activity

For example, the preosteoclasts obtained by the invention are prepared as 1×10^6 cell/well and cultured on 48 wells plate. In the cultivation, essential medium for mammalian cells, contains IL-3, IL-7, GM-CSF, eotaxin, eotaxin-2, eotaxin-3 or a supernatant of mitogen-stimulated peripheral blood mononuclear cells, is used. Various concentration of a candidate compound for therapeutic agents is added into the each well, and the prepared plate is cultured at 37° C., for 48–96 hours. At the end of the culture period, the adherent cells are fixed and stained with tartrate-resistant acid phosphatase (TRAP) which is the specific maker enzyme for osteoclasts. The multinucleate (N>3) and TRAP positive cell is counted as an osteoclast in each well. By calculating the result as IC_{50}, it is possible to evaluate the differentiation inhibitory activity of the candidate compound against the preosteoclasts.

(2) Measuring Method of Bone Resorption Inhibitory Activity

For example, the osteoclasts obtained by the invention are prepared as 1×10^6 cell/well and cultured on a dentine slice in the essential medium for mammalian cells 48-well culture plate. Various concentration of a candidate compound for therapeutic agents is added into the each well, and the cells are cultured at 37° C., for 48–96 hours. At the end of the culture period, the dentine slice is stained with hematoxylin. Osteoclasts resorb hydroxyappatite, and the resorbed parts (resorption pit) is visualized by the hematoxylin staining when the bone resorption is occurred. The formation of resorption pit on dentine slice is observed microscopically or an electron-microscopically. Otherwise, the change of calcium concentration in the culture supernatant can be assayed. By calculating the result as IC_{50}, it is possible to evaluate a bone resorption inhibitory activity of the candidate compound against the osteoclasts.

The inhibitory activity on the osteoclasts formation is becoming important as an indicator for assessing an antirheumatic activity. Consequently, a compound which has a differentiation inhibitory activity against preosteoclasts, is hopeful as an antirheumatic drug. Further, osteoporosis is caused by an excessive bone resorption. Thus, a compound with a bone resorption inhibitory activity against osteoclasts is hopeful as a therapeutic agent for osteoporosis.

EXAMPLE

Example 1

Isolation of Human Osteoclasts from Joint Fluid of Patients with Rheumatoid Arthritis

(1) Separation of a Cellular Fraction

Joint fluids were obtained from patients with rheumatoid arthritis. The joint fluids were kept in tubes at 4° C. The following procedures were generally under sterile conditions. The joint fluid, 1 ml to several tens ml, was added to equal volume of RPMI 1640 medium (Gibco BRL, #22400 or equivalent). The mixture was centrifugated at 1,000–2,000 rpm for 5 minutes at 4° C. to obtain a cellular fraction containing granulocytes and lymphocytes.

(2) Isolation of Preosteoclasts

The obtained cellular fraction was cultured in DMEM medium (Gibco BRL, #12430-21 or equivalent) supplemented with 10% (v/v) of fetal calf serum (FCS) in 5–7% CO_2-containing air at 37° C. for several weeks. During the culture period, all cells except preosteoclasts died out, and only preosteoclasts survived (FIGS. 1, 2).

(3) Preparation of Medium

The medium was prepared for the differentiation of preosteoclasts into osteiclasts . Four hundred ml of 400 ml of AIM-V medium (Gibco BRL, #87-0112) was supplemented with 60 ml of RPMI1640 medium (Gibco BRL, #22400), 40 ml of human T-STIM (10 BRMP/ml; BRMP, Biological Response Modifier Program Jurkat IL-2 reference reagent), 50 ml of 10% FCS (inactivated in advance by heating at 56° C. for 30 minutes), and antibiotics (100 U of penicillin and 100 μg/ml of streptomycin; Gibco BRL, #15140-015 or equivalent), and used as Medium A. Human T-STIM is a culture supernatant of human peripheral blood mononuclear cells stimulated with phytohemagglutinin (Human T-STIM with PHA, Becton Dickinson, #40045).

(4) Induction of Differentiation of Preosteoclasts Into Osteoclasts

The preosteoclasts obtained in Example 1(2) were stimulated with Medium A described in Example 1(3) at 37° C. for 48–96 hours. In the culture condition, the differentiation of preosteoclasts into osteoclasts was observed (FIG. 1, 2).

(5) Identification of the Cytokine Possesing a Differentiation-inducing Ability

Human T-STIM contains a variety of cytokines. In order to identify the cytokine which stimulates differentiation of preosteoclasts into osteoclasts, the differentiation was examined by modifycation the contents of Medium A described as previously in Example 1(3).

① Preparation of the Medium

The following 16 kinds of medium (B-R) were prepared by using the cytokines shown below, alone or a mixture thereof, instead of human T-STIM contained in Medium A (Example 1(3)). The other materials than human T-STIM, namely, AIM-V medium, RPMI 1640 medium, 10% FCS and antibiotics (100 U of penicillin and 100 μg/ml of streptomycin; Gibco BRL, #15140-015 or it's equivalent) were the same as those contained in Medium A.

Medium B: 0.5–5 ng/ml of recombinant human IL-1 (R & D Systems, #201-LB, #200-LA or equivalent)+400 ml of AIM-V medium+60 ml of RPMI 1640 medium+50 ml of FCS+antibiotics

Medium C: 50–200 U/ml of recombinant human IL-2 (R & D Systems, #202-IL, or equivalent)+400 ml of AIM-V medium+60 ml of RPMI 1640 medium+50 ml of FCS+antibiotics

Medium D: 2–10 ng/ml of recombinant human IL-3 (R & D Systems, #403-ML-010 or equivalent)+400 ml of AIM-V medium+60 ml of RPMI 1640 medium+50 ml of FCS+antibiotics

Medium E: 50–200 U/ml of recombinant human IL-4 (Genzyme, #2181-01 or equivalent)+400 ml of AIM-V medium+60 ml of RPMI 1640 medium+50 ml of FCS+antibiotics

Medium F: 10–20 ng/ml of recombinant human IL-6 (R & D Systems, #206-IL or equivalent)+400 ml of AIM-V medium+60 ml of RPMI 1640 medium+50 ml of FCS+antibiotics

Medium G: 10–20 ng/ml of recombinant human IL-6 (R & D Systems, #206-IL or equivalent)+100–300 ng/ml of recombinant human soluble IL-6 receptor (R & D Systems, #227-SR or it's equivalent)+400 ml of AIM-V medium+60 ml of RPMI 1640 medium+50 ml of FCS+antibiotics

Medium H: 5–20 ng/ml of recombinant human IL-7 (Genzyme, #1587-00 or equivalent)+400 ml of AIM-V medium+60 ml of RPMI 1640 medium+50 ml of FCS+antibiotics

Medium I: 1–4 ng/ml of recombinant human IL-11 (R & D Systems, #218-IL or equivalent)+400 ml of AIM-V medium+60 ml of RPMI 1640 medium+50 ml of FCS+antibiotics

Medium J: 2.5–100 ng/ml of recombinant human M-CSF (R & D Systems, #216-MC-010 or equivalent)+400 ml of AIM-V medium+60 ml of RPMI 1640 medium+50 ml of FCS+antibiotics

Medium K: 1–5 ng/ml of recombinant human GM-CSG (R & D Systems, #215-GM-010 or equivalent)+400 ml of AIM-V medium+60 ml of RPMI 1640 medium+50 ml of FCS+antibiotics

Medium L: 2–10 ng/ml of recombinant human IL-3 (R & D Systems, #403-010 or equivalent)+5–20 ng/ml of recombinant human IL-7 (Genzyme, #1587-00 or it's equivalent)+400 ml of AIM-V medium+60 ml of RPMI 1640 medium+50 ml of FCS+antibiotics

Medium M: 5–20 ng/ml of recombinant human IL-7 (Genzyme, #1587-00 or equivalent)+1–5 ng/ml of recombinant human GM-CSF (R & D Systems, #215-GM-010 or equivalent)+400 ml of AIM-V medium+60 ml of RPMI 1640 medium+50 ml of FCS+antibiotics

Medium N: 2–10 ng/ml of recombinant human IL-3 (R & D Systems, #403-ML-010 or equivalent)+5–20 ng/ml of recombinant human IL-7 (Genzyme, #1587-00 or equivalent)+1–5 ng/ml of recombinant human GM-CSF (R & D Systems, #215-GM-010 or equivalent)+400 ml of AIM-V medium+60 ml of RPMI 1640 medium+50 ml of FCS+antibiotics

Medium P: 10–1000 ng/ml of recombinant human eotaxin (R & D Systems, #220-EO or equivalent)+400 ml of AIM-V medium+60 ml of RPMI 1640 medium+50 ml of FCS+antibiotics

Medium Q: 10–1000 ng/ml of recombinant human eotaxin-2 (R & D Systems, #343-E2 or equivalent)+400 ml of AIM-V medium+60 ml of RPMI 1640 medium+50 ml of FCS+antibiotics

Medium R: 10–1000 ng/ml of recombinant human Eotaxin-3+400 ml of AIM-V medium+60 ml of RPMI 1640 medium+50 ml of FCS+antibiotics (Eotaxin-3 was found in the human genome sequencing data (H RG356E01) published by Washington University Genome Sequence Center, an exson sequence, which is considered to exhibit a significant homology but to encode a different chemokine protein, compared to known CC type chemokines. According to the sequence, cDNA of eotaxin-3 was obtained as shown in SEQ ID No.1. Recombinant baculovirus containing the sequence was prepared to infect insect cells, then the recombinant eotaxin-3 protein was purified from the culture supernatant.)

② Induction of the differentiation of preosteoclasts into osteiclasts

The preosteoclasts obtained by Example 1(2) were cultured in the Medium B-N described above at 37° C. for 48–96 hours. The differentiation of preosteoclasts into osteoclats was observed in the Medium D, H, K, M, N, P, Q and R. However, the differentiation was not occurred in the Medium B, C, E, F, G, I and J. From these results, it has become clear that IL-3, IL-7, GM-CSF, eotaxin, eotaxin-2, eotaxin-3 and the mixture thereof have an ability for inducing the differentiation of preosteoclasts. And, it was confirmed that IL-1, IL-2, IL-4, IL-6, IL-11 and M-CSF do not have an ability for inducing the differentiation of preosteoclasts.

On the differentiation requiring the presence of accessory cells, it has been known that IL-1, IL-6, IL-11, and M-CSF induce the differentiation of preosteoclasts into osteoclasts. But it has not been clear that the differentiation was induced by the result of the stimulation of preosteoclasts or accessory cells.

On the other hand, on the differentiation of preosteoclasts in this invention which does not require the presence of accessory cell, it becomes clear that IL-3, IL-7, GM-CSF, eotaxin, eotaxin-2, and eotaxin-3 have the differentiation-inducing ability. Furthermore, IL-1, IL-6, IL-11 or M-CSF does not effect to the differentiation.

Example 2

Isolation of Human Osteoclasts from Peripheral Blood of Healty Donors

(1) Separation of Cellular Fraction

Fifty ml to 200 ml of peripheral blood of healthy donors were collected in the presence of heparin or an alternative anticoagulant. Peripheral blood mononuclear cells (PBMC)

were obtained by a specific gravity centrifugation using a Ficoll-paque (Pharmacia Biotech). 10^7 cell/ml of PBMC were suspended in RPMI1640 medium containing 10% (v/v) FCS, then 1–1.5 ml/dish of the cell suspension was cultured on 60 mm culture dish at 37° C., in 5–7% CO_2-containing air, for 1–2 hours. After the culture, the non-adherent cells were rinsed out from the dish with RPIM1640 medium containing 10% (v/v) of FCS at 37° C. Adherent cells to the dish were washed with cold (4° C.), serum-free RPIM1640 medium, then the cells were collected as a peripheral blood monocytes (about 3–8% of total PBMC).

(2) Isolation of Preosteoclasts

The obtained monocytes ($0.5–1\times10^6$/ml) was cultured in DMEM medium (Gibco BRL, #12430-21 or equivalent) supplemented with 10% of FCS in 5–7% CO_2-containing air at 37° C. for several weeks likewise Example 1 (2). During the culture period, all cells except preosteoclasts died out, and only preostetoclasts survived. In cocultivation of the peripheral blood monoctyes and synovial nurse cells-derived rheumatoid arthritis subjects, the proliferation efficiency of the preosteoclasts increased. At the time, the same medium as Example 1 (2) was used.

(3) Induction of Differentiation

The preosteoclasts were stimulated with Medium A described in Example 1(3) at 37° C. for 48–96 hours. The differentiation of preosteoclasts was occurred and osteoclasts were obtained.

It was confirmed that the cells obtained at Example 1(4) and Example 2(3) were osteoclasts by the following testing examples.

Testing Example

(1) Morphologic Observation

The cells were stained by May-Giemsa solution, and were examined microscopically. The stained preosteoclasts and the stained osteiclasts are shown in FIG. 1 instead of photomicrograph. Form the result, it was confirmed that the preosteoclasts before the differentiation had a monocyte like morphilogy and the osteoclasts were multinucleated (over 3–100) giant cells.

(2) TRAP Stain

The preosteoclasts before the differentiation and the osteoclasts after the differentiation were stained by using a TRAP (tartrate-resistant acid phosphatase) staining kit (Sigma Co.) and examined by a microscope. The cells before the differentiation (preosteoclasts) and the cells after the differentiation (osteoclasts) are shown in FIG. 2 instead of photomicrograph. The figures shows that the cells before the differentiation (preosteoclasts) were positive for TRAP, and the cells after the differentiation (osteoclasts) were positive for TRAP especially around the nuclei.

(3) Bone Resorbing Activity

The preosteoclasts were cultured on dentine slices under the condition which cause the differentiation (Example 1(4)), then the dentine slices were stained with hematoxylin (Sigma Co.) and examined by a microscope. FIG. 3 shows the surfaces of the slice instead of the photomicrograph. The preosteoclasts before the differentiation had no effect on the dentine slices. But the osteoclasts after the differentiation resorbed calcium phosphate and the resorption pit on the slices were strongly stained with the dye. The dentine slices resorbed by the osteoclasts were examined by a scanning electron microscopy. FIG. 4 shows the figure instead of the scanning electron photomicrograph. The formation of the resorption pit is noted at the center of the figure. The resorption of calcium phosphate formed the pit and collagen fibers were exposed outside.

Futher, the preosteoclasts were differentiated on calcium phosphate sintering quartz discs (Osteologic™, Sumisho Pharma) and the discs were examined by a phase-contrast microscope. FIG. 5 shows the figure instead of the phase-contrast photomicrograph. The preosteoclasts before the differentiation had no effect on the calcium phosphate sintering quartz discs. But the resorption (the transparent area of the disc) by the osteoclasts after the differentiation was observed.

From the results shown in the testing examples, it was confirmed that the cells before the differentiation were preosteoclasts and the cells after the differentiation were osteoclasts.

Industrial Applicability

According to the invention, osteoclasts can be obtained from the same individual repeatedly, thus the pathophysiological or immunological investigation of the osteoclasts can be performed by using them. The screening of candidate compounds useful for treating metabolic bone diseases can be examined easily by using the osteoclast precurosor cells or osteoclasts of the invention. For example, it is effective to use a compound, which is obtained by the screening for bone resorption-inhibitory activity against the osteoclasts and shows a bone resorption-inhibiting ability against the osteoclasts, to treat the metabolic bone disease, including osteoporosis caused by an excessive bone resorption, decrease of bone quantity due to diabetes, and osteomalacia.

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Val Leu Leu Ala Ser Leu Leu Ser Leu His Leu Gly Thr Ala Thr Arg
10                  15                  20                  25

ggg agt gac ata tcc aag acc tgc tgc ttc caa tac agc cac aag ccc      150
Gly Ser Asp Ile Ser Lys Thr Cys Cys Phe Gln Tyr Ser His Lys Pro
                30                  35                  40

ctt ccc tgg acc tgg gtg cga agc tat gaa ttc acc agt aac agc tgc      198
Leu Pro Trp Thr Trp Val Arg Ser Tyr Glu Phe Thr Ser Asn Ser Cys
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tcc cag cgg gct gtg ata ttc act acc aaa aga ggc aag aaa gtc tgt      246
Ser Gln Arg Ala Val Ile Phe Thr Thr Lys Arg Gly Lys Lys Val Cys
        60                  65                  70

acc cat cca agg aaa aaa tgg gtg caa aaa tac att tct tta ctg aaa      294
Thr His Pro Arg Lys Lys Trp Val Gln Lys Tyr Ile Ser Leu Leu Lys
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Thr Pro Lys Gln Leu
90

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<213> ORGANISM: Homo sapiens

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            20                  25                  30

Cys Cys Phe Gln Tyr Ser His Lys Pro Leu Pro Trp Thr Trp Val Arg
        35                  40                  45

Ser Tyr Glu Phe Thr Ser Asn Ser Cys Ser Gln Arg Ala Val Ile Phe
    50                  55                  60

Thr Thr Lys Arg Gly Lys Lys Val Cys Thr His Pro Arg Lys Lys Trp
65                  70                  75                  80

Val Gln Lys Tyr Ile Ser Leu Leu Lys Thr Pro Lys Gln Leu
                85                  90
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What is claimed is:

1. A method for producing an osteoclast, comprising:

culturing an osteoclast precursor cell in the absence of accessory cells in a culture medium comprising one or more compound(s) selected from the group consisting of IL-3, IL-7, GM-CSF, eotaxin, eotaxin-2, and eotaxin-3, or in a culture medium comprising a culture supernatant of mitogen-stimulated peripheral blood mononuclear cells and

recovering or isolating an osteoclast;

wherein said osteoclast precursor cell is obtained by culturing a hematopoietic stem cell-derived cell obtained from peripheral blood or joint fluid in an essential medium for mammalian cells, optionally with added serum, in the absence of additional cytokine(s).

2. The method of claim 1, wherein said osteoclast precursor cell is obtained by culturing the cell obtained from joint fluid.

3. The method of claim 1, wherein said osteoclast precursor cell is obtained by culturing the cell obtained from peripheral blood.

4. The method of claim 1, wherein said culture medium comprises IL-3.

5. The method of claim 1, wherein said culture medium comprises IL-7.

6. The method of claim 1, wherein said culture medium comprises GM-CSF.

7. The method of claim 1, wherein said culture medium comprises eotaxin.

8. The method of claim 1, wherein said culture medium comprises eotaxin-2.

9. The method of claim 1, wherein said culture medium comprises eotaxin-3.

10. The method of claim 1, wherein said culture medium comprises a culture supernatant of mitogen-stimulated peripheral blood mononuclear cells.

11. The method of claim 1, wherein said culture supernatant is a culture supernatant of phytohemagglutinin-stimulated human peripheral blood mononuclear cells.

12. The method of claim 1, wherein said osteoclast precursor cell is obtained by culturing said hematopoietic stem cell-derived cell for 1–3 weeks.

13. The method of claim 1, wherein said essential medium for mammalian cells contains serum.

14. The method of claim 1, wherein said hematopoietic stem cell-derived cell is obtained from peripheral blood mononuclear cells.

15. The method of claim 1, wherein said hematopoietic stem cell-derived cell is obtained from a cellular fraction of joint fluid which contains granulocytes and lymphocytes.

16. The method of claim 1, wherein said hematopoietic stem cell-derived cell is cultured at a temperature of 35–37° C. in 5–7% CO_2-containing air for 1–3 weeks.

* * * * *

DNA Research vol. 112, 1 11 (2006)

Isolation and Expression Profiling of Genes Upregulated in the Peripheral Blood Cells of Systemic Lupus Erythematosus Patients

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Abstract

We have identified the genes whose expressions are augmented in the blood cells of the patients with systemic lupus erythematosus (SLE) using the 'stepwise subtraction' technique along with microarray analysis. The expression levels of these genes were assessed by quantitative real-time reverse transcription polymerase chain reaction (RT–PCR) in 31 SLE patients and 30 healthy controls. We found that the transcription levels of following eight genes were significantly increased in SLE patients; interferon (IFN)-α-inducible protein 27 (*IFI27*), IFN-α-inducible protein IFI-15K (*G1P2*), IFN stimulated gene 20 kDa (*ISG20*), epithelial stromal interaction 1 (*EPSTI1*), defensin-α (*DEFA3*), amphiregulin (*AREG*) and two genes of unknown function (BLAST accession nos *AL050200* and *AY358234* = *SLED1*). In comparison with idiopathic thrombocytopenic purpura (ITP), an organ-specific autoimmune disease, *IFI27*, *G1P2* and *SLED1* were preferentially upregulated in SLE. In contrast, *AREG* and *AL050200* were more highly expressed in ITP than in SLE. We correlated changes in gene expression and clinical/laboratory features of SLE and found that expression of *ISG20*, *EPSTI1* and *SLED1* are significantly correlated with lymphocyte counts. Genes linked to IFN are well known to influence SLE, but several other novel genes unrelated to IFN signaling we report here would be useful to understand the pathophysiology of SLE.

Key words: stepwise subtraction; microarray; SLE; ITP; interferon

1. Introduction

Systemic lupus erythematosus (SLE) is a systemic inflammatory autoimmune disease, characterized by production of multiple diverse autoantibodies against several self-antigens and resultant injury to various organ systems, including skin, joints, kidney and central nervous system. The pathogenesis of SLE is correlated with both genetic predispositions and environmental influences.[1] The contribution of these two factors may differ between individuals, but the resulting malfunctions in the immune system and the production of autoantibodies plays a pivotal role in the pathogenesis of SLE. Previous studies have revealed that symptoms resembling SLE appear in a variety of immunological disorders,[1–3] but the mechanisms of SLE pathogenesis are not known, and the cause of the diversity of symptoms is unclear.

Communicated by Mitsuo Oshimura

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Recent reports using cDNA/oligonucleotide array analysis on gene expression profiles in peripheral blood mononuclear cells (PBMC) from SLE patients have identified candidate genes responsible for SLE pathophysiology.[4 7] Several interferon (IFN) related genes are highly overexpressed in the peripheral blood and kidney glomeruli of SLE patients, supporting a central role for IFN in SLE.[8,9] Activation of the IFN-α pathway defines a subgroup of SLE patients whose condition is characterized by increased disease severity.[10] In some SLE patients, activated T cells seem to resist anergy and apoptosis by markedly upregulating and sustaining cyclooxygenase-2 expression.[11] However, the pathological significance of these changes in gene expression remains controversial and whether the changes are specific to SLE is open to question.[12,13]

We have developed a novel variation of the subtractive hybridization technique called stepwise subtraction for comprehensive gene discovery, wherein the subtraction process is systematically repeated in a stepwise manner to isolate essentially all of the genes whose expression is specifically upregulated relative to a control population.[14] This technique has proven useful in the discovery of genes specifically expressed during cancer metastasis, meiosis and spermatogenesis.[14 16] This technique complements cDNA microarray analysis because it can isolate novel genes that were not identified by microarray screening.[17] Here, we applied the stepwise subtraction method together with microarray analysis to identify a set of genes differentially expressed in SLE, including several candidates which have not been previously associated with SLE. The expression of these genes was confirmed and quantified with real-time reverse transcription polymerase chain reaction RT–PCR and the expression levels were compared to indicators of SLE pathology. In addition, we investigated patients with idiopathic thrombocytopenic purpura (ITP), which is an organ-specific autoimmune disease. We compared the levels of gene expression in SLE patients with those in ITP patients and identified changes in gene expression that are specific to each condition.

2. Materials and Methods

2.1 Human subjects: patients and healthy controls

Blood was obtained from 133 SLE patients (8 men and 125 women) and eight healthy controls (4 men and 4 women) (Supplementary Table 1, set A), for stepwise subtractive hybridization and microarray analysis. For real-time RT–PCR, 31 SLE patients (1 man and 30 women) were randomly selected from set A. Independently, blood samples from 25 ITP patients (8 men and 17 women), and 30 healthy controls (all women) were analyzed (Supplementary Table 1B, set B). Written informed consent was obtained from all participating subjects. This study was performed according to the guidelines of Osaka University Graduate School of Medicine, which abides by the Helsinki Declaration on ethical principles for medical research involving human subjects. All SLE patients fulfilled the American College of Rheumatology classification criteria for SLE.[18] ITP patients were diagnosed based on idiopathic thrombocytopenia (platelets $<100 \times 10^9$/l) and megakaryocytic hyperplasia or normoplasia in the bone marrow, when other causes had been excluded. Clinical manifestations and laboratory features of the SLE patients were also examined, including the SLE disease activity index (SLEDAI) score,[19] counts of peripheral lymphocytes, dosages of oral glucocorticoid or immunosuppressants, ages of the patients and length of time from disease onset.

2.2 RNA isolation and multiple tissue cDNA panels

Heparinized venous blood (10 ml) was mixed with an equal volume of 2% dextran/saline solution and incubated at room temperature for 30 min to precipitate red blood cells. PBMC in the supernatant were purified by density-gradient centrifugation on Percoll (density = 1.064 g/ml). Total RNA was extracted from the PMBC pellets by adding guanidine-thiocyanate solution and the samples were used for cDNA library preparation and subtractive hybridization or acid guanidinium-phenol-chloroform extraction for real-time RT–PCR.[20] To analyze the expression pattern of some SLE-upregulated genes, PCR was performed on multiple tissue cDNA panels (Clontech Laboratories, Palo Alto, CA, USA) using the coding sense and antisense primers for 25–40 cycles (see Figure 5) at 95°C for 30 s, 55°C for 30 s and 72°C for 1 min. Ex*Taq* DNA polymerase was purchased from TaKaRa Co. Ltd (Otsu, Japan).

2.3 Preparation of cDNA library and stepwise subtraction

An SLE cDNA library was constructed from PBMC of 133 SLE patients (Supplementary Table 1, Set A) using the linker-primer method with a pAP3neo vector, as described previously.[20] We also prepared mRNA from PBMC of healthy controls (Supplementary Table 1, Set A) and biotinylated the mRNA with photobiotin to perform cDNA subtractions.[20] To analyze the quality of the first-stage subtracted cDNA library, cDNA inserts from 480 randomly selected cDNA clones were restriction digested with *Eco*RI and *Not*I, and the fragments isolated from 1% agarose gels. The fragments were then ^{32}P-labeled to use as probes for northern analysis to identify SLE specific clones (see Figure 1B). The stepwise subtraction of this cDNA library was performed as described previously.[14] We isolated almost all of the SLE specific clones included in the original cDNA library from the first and second subtraction, since only a few independent cDNA clones were detected in the third subtraction.

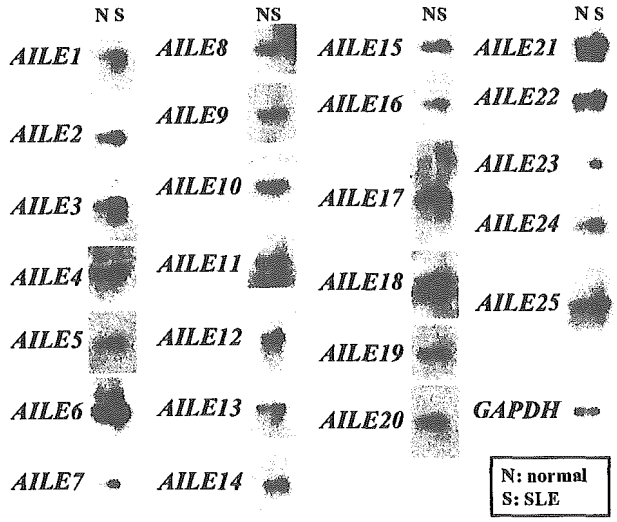


Figure 1. Northern blots of individual *AILE* cDNA clones to compare the expression levels of the genes in SLE patients and normal controls (see Supplementary Table 1, Set A). A northern blot with *GAPDH* probe is shown as a loading control.

The DNA sequences of the SLE specific clones were determined using an automated DNA sequencer (ABI PRISM 377; Applied Biosystems, Foster City, CA, USA).

2.4 DNA microarray analysis

The quality of the RNA samples was examined using the RNA 6000 Nano LabChip Kit (p/n 5065-4476) on the Agilent 2100 Bioanalyzer (G2940BA; Agilent Technologies, Inc., Palo Alto, CA, USA). Total RNA (500 ng) from PBMC of 133 SLE patients or 8 healthy volunteers was reverse-transcribed using oligo-dT primers containing the T7 RNA polymerase promoter sequence, and the cDNAs were then subjected to *in vitro* transcription using T7 RNA polymerase to label cDNAs with Cy3 or Cy5 (CyDye, Amersham Pharmacia Biotech, Piscataway, NJ, USA). Cy-labeled cRNA from SLE patients (1 μg) was mixed with the same amount of reverse color Cy-labeled product from an equal amount of pooled cRNA from healthy volunteers. Labeled cRNAs were fragmented to an average size of approximately 50–100 nt by heating at 60°C in the presence of 10 mM $ZnCl_2$, and the samples were then added to a hybridization buffer containing 1 M NaCl, 0.5% sodium sarcosine, 50 mM MES (pH 6.5) and formamide to a final concentration of 30%, in a final volume 3 ml. Hybridizations with the Agilent's whole human genome microarray (Hu44K) were conducted at 40°C. Sequences for microarrays were selected from RefSeq (a collection of non-redundant mRNA sequences; http://www.ncbi.nlm.nih.gov/LocusLink/refseq.html) and from expressed sequence tag (EST) contigs (http://www.phrap.org/est_assembly/human/gene_number_methods.html). Each mRNA or EST contig was represented on the Hu44K microarray by a single 60mer oligonucleotide chosen by the oligonucleotide probe design program. After hybridization, slides were washed and scanned using an Agilent confocal laser scanner (G2565BA). Fluorescence intensities on scanned images were quantified, corrected for background noise and normalized. Fluorophore reversal (dye swap) duplicates were used in two-color DNA microarray experiments.

2.5 Quantitative real-time PCR

Relative quantitation with real-time RT–PCR was performed using an ABI PRISM 7900 (PE Applied Biosystems, Foster City, CA, USA) and the Assay-on-Demand TaqMan probe and relevant primers, according to the manufacturer's instructions. Total RNA (500 ng) obtained by acid guanidinium-phenol-chloroform extraction was reverse-transcribed using the High Capacity cDNA Archive Kit (ABI). The cDNA was used as a template for PCR in a 50 μl reaction using 2× Master Mix according to the manufacturer's instructions (TaKaRa). PCR consisted of initial denaturation (95°C, 10 min), then 40 cycles of denaturation (95°C, 15 s) and annealing/extension (60°C, 1 min). Each sample was assayed in quadruplicate and the median threshold cycle (CT) values were used to calculate the fold change (FC) between patient and control samples. Standard deviation and standard error were also calculated. A standard curve from the amplification data for each primer was generated using a dilution series of total RNA from PBMC as templates, FC values were normalized to GAPDH levels using the standard curve method according to the manufacturer's protocol.

2.6 Purification of T cells and B cells, and their characteristics

PBMC from three SLE patients were isolated by density-gradient centrifugation with Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). T or B cells were purified by magnetic cell sorting (MACS) using a StemSep™ Kit (Stem Cell Technologies Inc., British Columbia, Canada) as described elsewhere (Thomas et al., 1989). We found that more than 95 or 93% of T or B cells purified by MACS were CD3 positive or CD19 positive, respectively. Purified CD3/CD19 positive populations were analyzed by flow cytometry (FACScan Cytometer, Becton Dickinson Immunocytometry System, Mountain View, CA, USA).

2.7 Statistical analysis

Significant differences were determined using the Mann–Whitney *U*-test or Spearman's rank correlation. Data are presented as the mean ± standard deviation (SD). $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 *Identification of SLE specific genes by stepwise subtraction and DNA microarray analysis*

To identify the putative SLE specific genes that are upregulated in PBMC of SLE patients compared to healthy volunteers, we applied stepwise subtractive hybridization.[14] Briefly, we prepared a pooled cDNA library using mRNA from 133 SLE patients (Supplementary Table 1, Set A) using the linker-primer method with a pAP3neo vector. Subtractive hybridization used biotinylated mRNA from 8 healthy volunteers (4 males and 4 females; Set A), generating the first subtracted cDNA library, as described previously.[20] The SLE-upregulated genes were identified by northern blot analysis using DNA inserts from randomly selected cDNA clones of the subtracted cDNA library. We performed the stepwise subtraction three times. Since we did not obtain any new SLE-upregulated genes in the third subtracted cDNA library (data not shown), the analysis was terminated at this point. We identified 25 SLE-upregulated genes by northern blot analysis (Figure 1) and named them *AILE* genes (augmented expression in SLE). As listed in Table 1. *AILE1* (*G0S2*) is a lymphocyte G_0/G_1 switch gene whose expression may be required to commit cells to enter the G_1 phase of the cell cycle.[21] *AILE4* (*IFI-15K*) and *AILE11* (*ISG20*) are IFN-inducible genes. *AILE8, AILE9, AILE14* and *AILE18* are human leukocyte antigen (HLA) related genes. *AILE6, AILE24* and *AILE25* are erythroblast related genes, whereas *AILE19* and *AILE20* are thymosin related genes.

We also performed DNA microarray analysis using the Agilent Hu44K array with the same samples of pooled RNA obtained from SLE patients and healthy volunteers. The list of the genes that were upregulated 7.8-fold or more in SLE patients relative to controls (top 50) is shown in Table 2. Five IFN-α-inducible genes (IFIG) (*AK095039, IFIT1, IFIT4, IFI44* and *G1P2*) were identified as SLE-upregulated genes. Notably, *G0S2* (*AILE1*), *G1P2* (*AILE4*), *JunB* (*AILE21*) and *PRG1* were identified as SLE-upregulated genes by both stepwise subtraction and microarray analysis. *AY358224*, which showed the most significant difference between SLE patients and normal volunteers in the microarray screening, encodes an uncharacterized protein. Because *AY358224* showed SLE-dependent upregulation we named it *SLED1* (see below).

3.2 *Expression profiles of SLE-upregulated genes in individual SLE patients*

To determine whether upregulation of each of these genes is widespread in SLE or occurs in only a few patients, we performed quantitative real-time RT–PCR using individually prepared RNA samples from the SLE patients and healthy controls. We selected 31 SLE patients from the same hospital (Supplementary Table 1, Set B) and 30 young females (age 18–20) as controls, in addition to the original controls (Set A). We tested 16 *AILE* genes (Table 1) and 6 SLE-upregulated genes (Table 2) for SLE specific upregulation by semi-quantitative PCR. We did not test the genes that encoded ribosomal proteins, hemoglobin gamma, ferritin, MLA-F or proteoglycan 1, because they are not considered to be causative for SLE. As shown in Supplementary Figure 1, the standard deviation for quadruplicate measurements from the same individual was small, and this reproducibility indicates that the values provide a reliable measure of gene expression levels.

Table 1. List of *AILE* genes identified by stepwise subtraction

Gene name	Accession no.	Sequence description
AILE1	NM_015714	Lymphocyte G_0/G_1 switch gene (G0S2)
AILE2	NM_002965	S100 calcium binding protein A9 (calgranulin B) (S100A9)
AILE3	AL050290	*Uncharacterized (DKFZp586G1923)*
AILE4	NM_005101	*IFN, alpha-inducible protein (IFI-15K) (G1P2)*
AILE5	NM_004084	*Defensin, alpha 1, myeloid-related sequence (DEFA1)*
AILE6	NM_000518	Hemoglobin, beta (HBB)
AILE7	NM_005217	*Defensin, alpha 3, neutrophil-specific (DEFA3)*
AILE8	M26038	MHC class II DR beta mRNA
AILE9	M84375	MHC class I antigen (HLA)
AILE10	AC090936	**Unknown (RP11-731I21)**
AILE11	NM_002201	*IFN stimulated gene 20 kDa (ISG20)*
AILE12	BC016369	**Unknown (MGC27165)**
AILE13	NM_002923	Regulator of G-protein signaling 2, 24 kDa (RGS2)
AILE14	NM_018950	Major histocompatibility complex, class I, F (HLA-F)
AILE15	NM_000977	Ribosomal protein L13 (RPL13)
AILE16	AC010890	**Unknown (RP11-449L24)**
AILE17	AL450322	**Unknown (RP11-184A2)**
AILE18	NM_004048	Beta-2-microglobulin (B2M)
AILE19	NM_002823	Prothymosin, alpha (PTMA)
AILE20	NM_021109	Thymosin, beta 4, X-linked (TMSB4X)
AILE21	NM_002229	Jun B proto-oncogene (JUNB)
AILE22	NM_002727	Proteoglycan 1, secretory granule (PRG1) ≒BC022313
AILE23	AC095053	**Unknown (RP11-352E8)**
AILE24	NM_000184	Hemoglobin, gamma G (HBG2)
AILE25	NM_002032	Ferritin, heavy polypeptide 1 (FTH1)

Unknown genes are shown in boldface. The genes that were also identified as SLE-upregulated genes by microarray screening (see Supplementary Table 3) are underlined. The sequence descriptions of genes that displayed either SLE or ITP specific upregulation are shown in italics.

Table 2. List of SLE-upregulated genes identified by DNA microarray analysis

Accession no.	Fold change[a]	Seqence description
AY358224	54.29	**Unknown (UNQ9368) = SLED1**
NM_001964	44.69	Early growth response 1 (EGR1), mRNA
AK025198	24.08	**Unknown (FLJ21545)**
NM_000584	23.24	Interleukin 8 (*IL8*)
NM_080657	20.65	Viperin (cig5)
BC002646	20.28	v-jun sarcoma virus 17 oncogene homolog (avian)
AK095039	17.97	Similar to IFN, alpha-inducible protein 27(IFI-27')
NM_005532	17.86	*IFN, alpha-inducible protein 27 (IFI27)*
NM_016323	17.31	Cyclin-E binding protein 1 (CEB1)
THC1854524	16.82	**Unknown**
A32P44932	16.12	**Unknown**
BC017969	15.33	Viperin (MGC:24122)
A24P917810	15.28	**Unknown**
NM_015714	14.82	Lymphocyte G_0/G_1 switch gene (G0S2), mRNA
AL831953	14.2	**Unknown (DKFZp667P0410)**
NM_001549	13.44	IFN-induced protein with tetratricopeptide repeats 4 (IFIT4)
A24P112542	13.34	**Unknown**
BC013734	12.62	Prostaglandin-endoperoxide synthase 2
NM_001657	12.32	*Amphiregulin (schwannoma-derived growth factor) (AREG)*
NM_003864	11.52	Sin3-associated polypeptide, 30 kDa (SAP30)
NM_017762	11.41	**Unknown (FLJ20313)**
NM_004233	11.36	CD83 antigen
NM_001781	11.32	CD69 antigen (p60, early T cell activation antigen)
NM_006417	11.25	IFN-induced protein 44 (IFI44)
AK022348	10.75	Weakly similar to CDC 48 homolog MJ1156
NM_020529	10.67	NFKBIA[b]
THC1948392	10.59	**Unknown**
AK091799	10.3	**Unknown (FLJ34480)**
THC1924602	9.46	**Unknown**
NM_005101	9.42	IFN, alpha-inducible protein (clone IFI-15K) (G1P2)
NM_033027	9.35	AXIN1 upregulated 1 (AXUD1), mRNA
NM_032354	9.31	**Unknown (MGC10744)**
AF193059	9.19	SP1224
NM_033255	9.13	*Epithelial stromal interaction 1 (breast) (EPSTI1)*
AV756170	9.1	**Unknown**
S69873	8.88	Mutant DNA polymerase beta
AK097130	8.81	**Unknown**
NM_017742	8.76	Zinc finger, CCHC domain containing 2 (ZCCHC2)
NM_006026	8.57	H1 histone family, member X (H1FX)
AL133570	8.55	**Unknown (from clone DKFZp434L201)**
BC006340	8.34	**Unknown (IMAGE:4079754)**
NM_001548	8.31	IFN-induced protein with tetratricopeptide repeats 1 (IFIT1)
AL137464	8.27	**Unknown (DKFZp434E1722)**
NM_002616	8.15	Period homolog 1 (*Drosophila*) (PER1)
NM_004556	8.15	NFKBIA[c]
NM_000051	8.15	Ataxia telangiectasia mutated (ATM), transcript variant 1
BC022313	8.11	Proteoglycan 1, secretory granule (PRG1)
AL049991	7.84	**Unknown (DKFZp564G222)**
AF000018	7.81	Adapter protein
NM_000201	7.76	Intercellular adhesion molecule 1 (CD54)(ICAM1)

Unknown genes are shown in boldface. Genes also identified by stepwise subtraction are underlined. The sequence descriptions of genes that displayed either SLE or ITP specific upregulation are shown in italics.

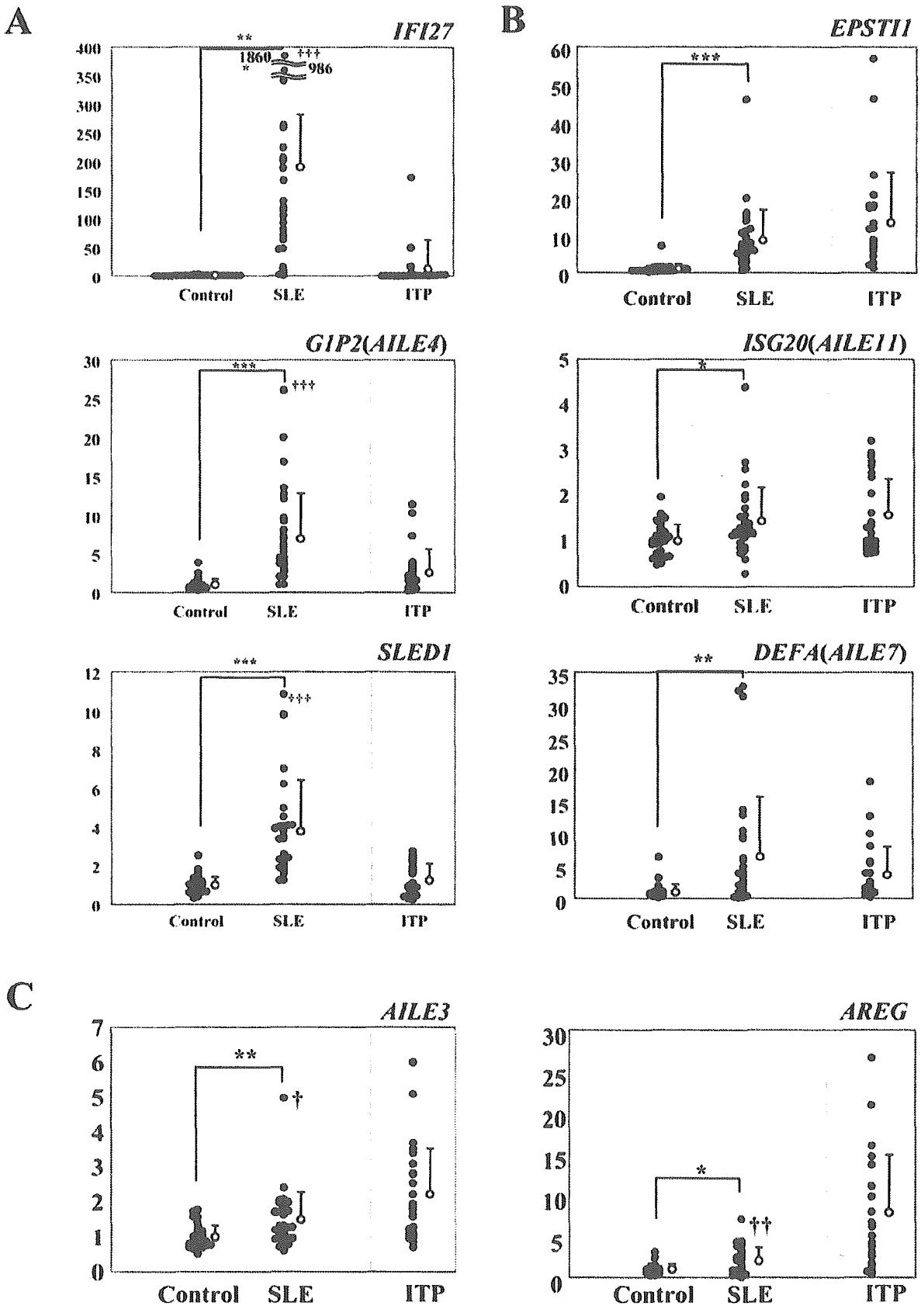
[a]Difference in expression level between SLE patients and healthy volunteers (Set A).

[b]NFKBIA = Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha.

[c]NFKBIE = Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, epsilon.

As shown in Figure 2, the *IFI27*, epithelial-stromal interaction 1 (*EPSTI1*), *G1P2* (*AILE4*), *ISG20* (*AILE11*), *SLED1* and *DEFA3* (*AILE7*) genes showed enhanced expression in many of the SLE patients. *IFI27*, *G1P2* and *ISG20* are IFIG; which is consistent with previous work showing that IFN-α is the predominant stimulus for FIG expression in lupus.[9] Moreover, we found that expression of defensin-3α (*DEFA33*), which is a major product of immature granulocytes and has antimicrobial activity, is enhanced in the PBMC of many SLE patients. *EPSTI1* is one of the upregulated genes in invasive breast carcinomas,[22] but this is the first report correlating upregulation of *EPSTI1* with SLE.

Expression of *AILE1* (*G0S2*) was enhanced in many SLE patients compared to the original 8 healthy controls (Set A), as shown in Supplementary Figure 2. These data are consistent with the northern blot analysis showing a dramatic upregulation of *AILE1* in SLE patients. However, when expression levels were compared to those of eight Set B controls, expression levels were similar to SLE patients. When we performed another series of real time RT–PCR assays with 30 Set B controls, they showed slightly enhanced expression relative to SLE patients. Thus, changes in *G0S2* expression do not appear to be correlated to SLE. Other tested genes did not show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IFI27
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Control
SLE
ITP

any significant increase as compared to healthy controls (Supplementary Figure 3).

3.3. *Comparison with ITP*

We next compared the expression levels of these genes in each SLE and ITP patient, and found three trends. Type A genes, comprised of *IFI27*, *G1P2* and *SLED1*, are upregulated in SLE relative to ITP (Figure 2A). These genes can be used as SLE specific gene markers to distinguish SLE from ITP. Type B genes, which include *EPSTI1*, *DEFA3* and *ISG20*, showed similar levels of expression in both SLE and ITP (Figure 2B). These genes can be used as gene markers to identify both SLE and ITP. Type C genes, consisting of *AILE3* and amphiregulin (*AREG*), are more conspicuously upregulated in ITP than SLE (Figure 2C). These genes can be used as ITP specific gene markers to distinguish ITP from SLE.

3.4. *Correlation analysis*

The correlations among expression levels of these genes showed that the genes linked to IFN (*IFI27*, *G1P2* and *ISG20*) displayed strong positive correlations in upregulation of gene expression (Figure 3A). *EPSTI1* expression also showed a strong positive correlation with *G1P2* and *ISG20* expression, suggesting that *EPSTI1* is somehow correlated with IFN relevant events. *SLED1* expression displayed a weak but significant positive correlation with *ISG20* expression (Figure 3B).

Next, we examined correlations of these genes with clinical manifestations, including the SLE disease activity index (SLEDAI), numbers of fulfilled classification criteria for SLE (NFCCS), presence or absence of lupus nephritis, counts of peripheral lymphocytes, dosages of oral steroid, ages of the patients and length of time since disease onset. We found significant negative correlations between lymphocyte counts and expression levels of *ISG20*, *EPSTI1* and *SLED1* (Figure 4A). We also found a reasonably good negative correlation between the NFCCS and the expression level of *AREG* (Figure 4B). Other clinical symptoms showed no significant correlations.

3.5. *Expression pattern in PBMC*

To analyze the expression pattern in human blood cell fractions of SLE-upregulated genes whose expressions are enhanced in many of the SLE patients (Figure 2A and B), we performed RT–PCR on multiple tissue cDNA panels (MTC from Clontech) and blood cell RNA of SLE patients (Figure 5). RT–PCR identified *IFI27* and *G1P2* genes as being expressed ubiquitously in most of the MTC blood cell fractions (Figure 5, lanes 1–9) and in SLE (Figure 5, lanes 13–15). The expression of *SLED1* is weak and localized to specific cell subsets, namely, monocytes (lane 4) and T and B cells (lanes 2–4). This result is consistent with the observation of *SLED1* expression in the whole blood fraction (total blood leukocytes) of an SLE patient (lane 13). *EPSTI1* was expressed in most cell types, except for activated $CD4^+$ T cells (lane 8), and was detected prominently in the total blood leukocyte fraction (lane 13) in SLE patients, suggesting that the dramatically increased expression (5–50-fold) of the *EPSTI1* gene (Figure 2B) results from monocytes rather than from B or T cells. *ISG20* is expressed in T and B cells but not in monocytes (lane 4), similar to SLE patients (lanes 14 and 15). *DEFA3* is expressed in suppressor T cells (lane 2), B cells (lane 5), and more prominently in other blood cells (lane 1), but expression of *DEFA3* in T and B cells increased in SLE patients (lanes 13–15). These results indicate that the expression pattern of these genes are not largely altered in SLE patients

4. Discussion

In the present study, we comprehensively isolated and analyzed the expression levels of genes that are upregulated in the PBMC of SLE patients, using stepwise subtractive hybridization in combination with oligonucleotide microarray analysis. This study identified many novel SLE-upregulated genes, in addition to *IFN* responsive genes, such as *IFI27*, *G1P2* and *ISG20*, which had been previously identified as upregulated in SLE using microarray analysis.[4 6] The importance of the type I IFN system in the etiology of SLE has garnered much attention.[8,23] The serum levels of IFN-α, a major effector in response to viral infection, is correlated with SLE disease activity, and IFN-α therapy sometimes produces autoimmune side effects, which resemble genuine SLE, including production of autoantibodies.[24] However, we did not isolate the transcript for IFN itself as an SLE-upregulated gene. The major IFN-producing cells, i.e. natural IFN-α producing cells (NIPC), are continuously activated in SLE, producing IFN.[25] The activator for NIPC remains to be elucidated but candidates are autoantibodies, unmethylated CpG motifs, or the presence of

Figure 2. Expression levels of SLE-upregulated genes. (**A**) The expression levels of the genes that are more highly upregulated in SLE than ITP. This group includes *IFI27*, IFN-α-inducible protein *G1P2* (*AILE4*) and *SLED1* (*AY358224*). Note the scale breaks for *IFI27* SLE samples. (**B**) The expression levels of the genes that are similarly upregulated in both SLE and ITP. This group includes *EPSTI1*, *DEFA3* and *ISG20*. (**C**) The expression levels of the genes that are more highly upregulated in ITP than SLE. This group includes *AILE3* and *AREG*. Filled circles denote the mean value of samples analyzed in quadruplicate from each individual. The open circle and bar signify the average + SD for each group, i.e. control, SLE or ITP. $^*P < 0.05$; control versus SLE, $^{**}P < 0.01$; control versus SLE, $^{***}P < 0.001$; control versus SLE, $^{\dagger}P < 0.05$; SLE versus ITP, $^{\dagger\dagger}P < 0.01$; SLE versus ITP, $^{\dagger\dagger\dagger}P < 0.001$; SLE versus ITP.

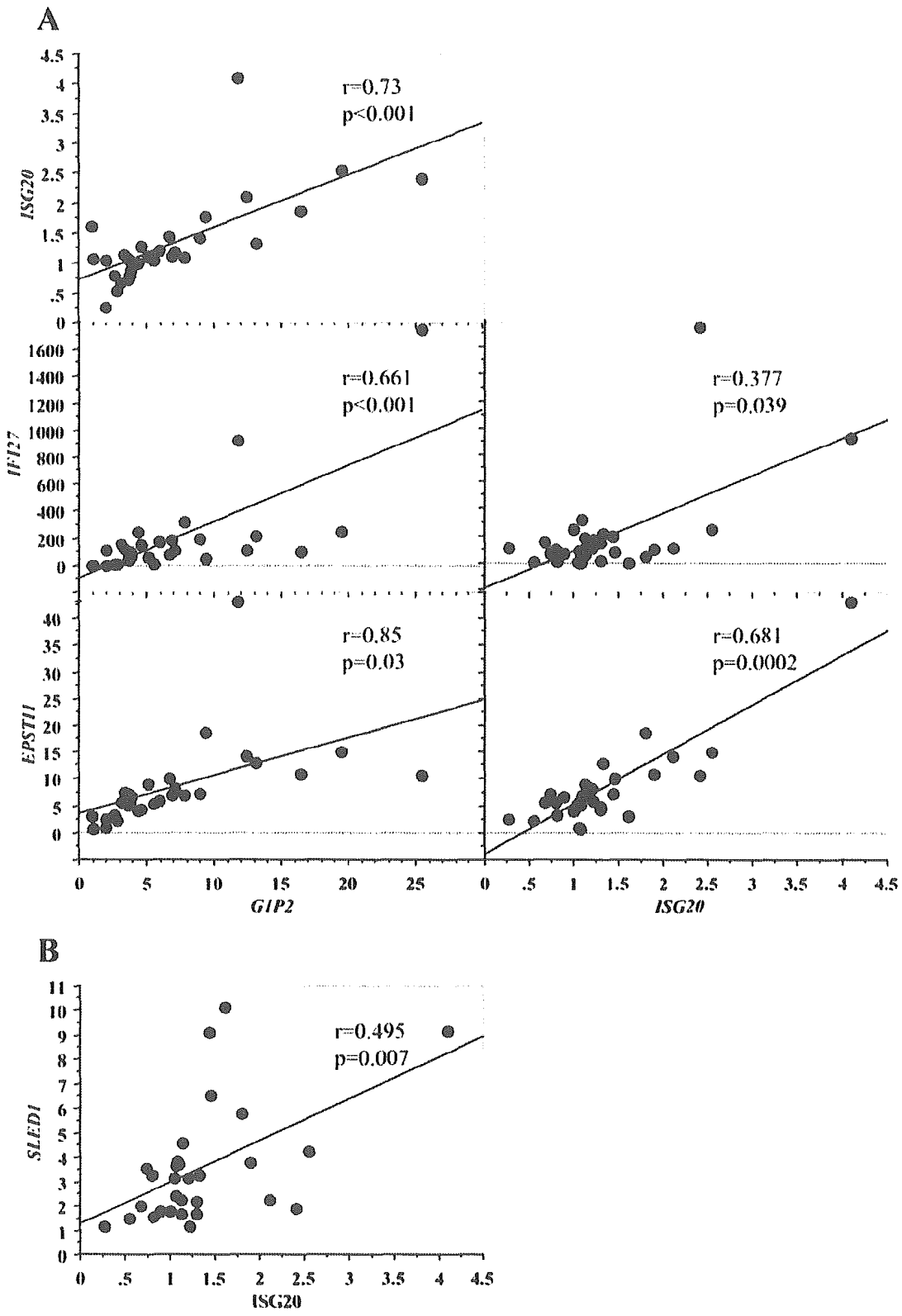

Figure 3. Positive correlations among expression of SLE upregulated genes. (**A**) Expression of *IFI27* showed a strong positive correlation with *G1P2* ($r = 0.661$) and a weak correlation with *ISG20* ($r = 0.377$) expression. In addition, *EPSTI1* expression showed a strong positive correlation with that of *G1P2* and *ISG20* ($r = 0.85$ and 0.681, respectively). (**B**) *SLED1* expression displayed a weak positive correlation with *ISG20* expression ($r = 0.495$). All correlations were significant ($P < 0.05$).

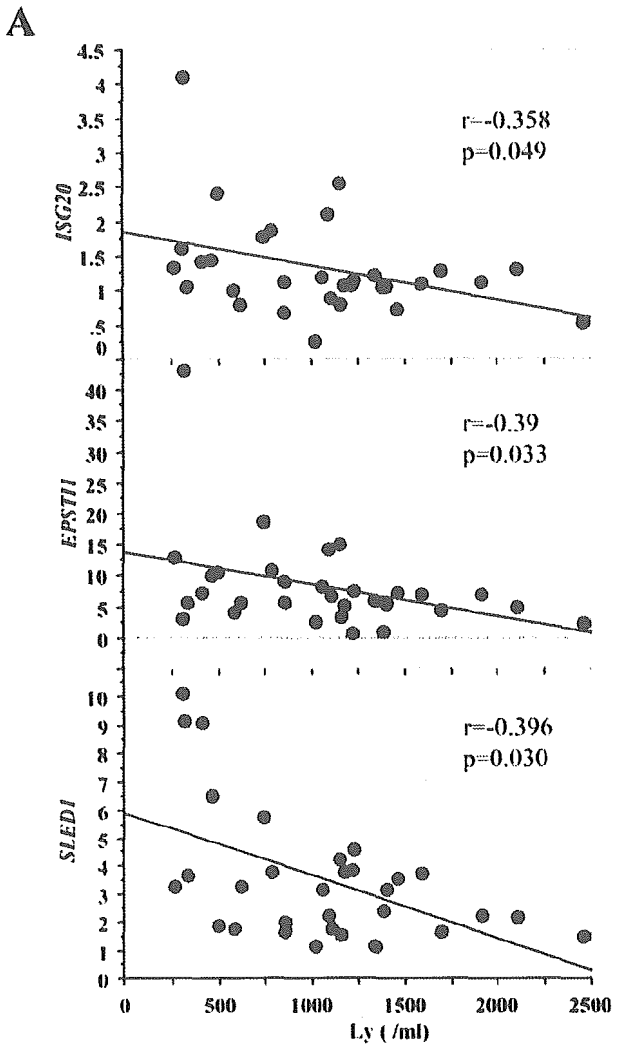

Figure 4. Correlation between the levels of gene expression and clinical data. (**A**) Significant negative correlations were observed between lymphocyte (Ly) counts and the levels of *ISG20* ($r = -0.358$), *EPSTI1* ($r = -0.39$) and *SLED1* ($r = -0.396$). (**B**) A negative correlation was also detected between numbers of SLE disease criteria and the level of *AREG* ($r = -0.366$). All correlations were significant ($P < 0.05$).

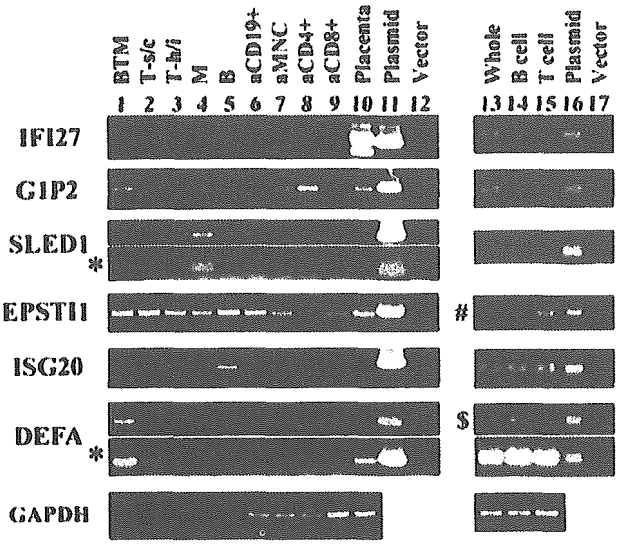

Figure 5. RT–PCR amplification of *IFI27*, *EPSTI1*, *G1P2*, *ISG20*, *SLED1*, *DEFA3*, *AILE3* and *AREG*. *GAPDH* was amplified as a loading control. The multiple tissue cDNA panel for human blood fractions (MTC, Clontech) (lanes 1–12) and SLE patients' blood fractions (lanes 13–15) were examined. 30 cycles of amplification were used for PCR, except as noted at the right of the panels: (#) 40 cycles, (*) 35 cycles or ($) 25 cycles. Lane 1, mononuclear cells (B, T cells and monocytes). Lane 2, resting CD8+ cells (T-suppressor/cytotoxic). Lane 3, resting CD4+ cells (T-helper/inducer). Lane 4, resting CD14+ cells (monocytes). Lane 5, resting CD19+ cells (B cells). Lane 6, activated mononuclear cells. Lane 7, activated CD4+ cells. Lane 8, activated CD8+ cells. Lane 9, activated CD19+ cells. Lane 10, human placenta control cDNA. Lane 11, plasmid harboring tested cDNA insert (size control). Lane 12, vector alone (negative control). Lane 13, whole blood cells of SLE patients. Lane 14, B cell fraction of SLE patients. Lane 15, T cell fraction of SLE patients. Lane 16, plasmid carrying relevant cDNA (positive control). Lane 17, vector alone (negative control).

necrotic or apoptotic cells. NIPC, which are reported to resemble immature dendritic cells in phenotype,[26] are a very minor component of PBMC. This may be why IFN mRNA was not detected.

We identified *DEFA3*, a major product of immature granulocytes, as an SLE-upregulated gene, which supports the previous data.[5] Here, we found that *DEFA3* was also upregulated in ITP. *DEFA3* was also upregulated in PBMC of rheumatoid arthritis patients.[27] Thus, *DEFA3* upregulation might be a general feature of autoimmune diseases. *EPSTI1* expression is enhanced in breast cancer upon direct interaction between tumor cells and stromal cells in the tumor environment assay.[22] *EPSTI1* is also upregulated in small intestine, spleen, salivary gland, testes and placenta, though its function remains to be elucidated.

AREG is a heparin-binding, heparin-inhibited member of the epidermal growth factor family and an autocrine growth factor for human keratinocytes. AREG plays an important role in psoriatic hyperplasia, and inhibition of AREG activity could be an efficacious therapeutic strategy for psoriasis.[28] Our results suggest that inhibition of

AREG activity may also be a therapeutic strategy for SLE or ITP. *AILE3* encodes an uncharacterized protein that belongs to the acetyltransferase family. *SLED1* encodes a small protein originally identified by the secreted protein discovery initiative (SPDI) (Genentech, Inc., CA, USA) as a secreted or transmembrane protein. Characterization of these proteins remains for future studies.

We classified the SLE upregulated genes by comparison to ITP and identified three groups. The genes which are more highly upregulated in SLE than ITP (*IFI27*, *G1P2* and *SLED1*), might influence systemic inflammation. Among them, IFI27 is most significantly upregulated. Genes which displayed the same levels of transcriptional upregulation in SLE and ITP (*EPSTI1*, *ISG20* and *DEFA3*), might be genes that are generally upregulated in autoimmune diseases. Genes showing more enhanced expression in ITP than SLE (*AILE3* and *AREG*), might be correlated with the organ-specific destruction of thrombocytes.

Taken together, we identified several novel SLE-upregulated genes whose expression profiling may provide a useful measure of the pathophysiology of SLE. Using the combination of stepwise subtractive hybridization and microarray analysis, the genes we detected should be very specifically correlated with SLE. Genes linked to IFN are well known to influence SLE, but here we isolated several other novel genes unrelated to IFN signaling. Further investigation is needed to clarify their roles in the pathogenesis of SLE.

Acknowledgements: The authors thank the patients and healthy volunteers who participated in this study. The authors also thank Ms Tomoko Motoyama for technical assistance, Dr Katsuhiko Ishihara for technical advice and Dr Patrick Hughes for critically reading the manuscript. The authors also thank Dr Daisuke Okuzaki of DNA-chip Development Center for Infectious Diseases (RIMD, Osaka University) for technical advice. This work was supported by Innovation Plaza Osaka of Japan Science and Technology Agency (JST), and grants-in-aid for Scientific Research on Priority Areas, Scientific Research (S), Exploratory Research and Science and Technology Incubation Program in Advanced Regions, from the Ministry of Education, Science, Sports and Culture.

Supplementary material: Supplementary material is available online at www.dnaresearch.oxfordjournals.org.

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