Large Scale Gene Expression Analysis of Osteoclastogenesis in Vitro and Elucidation of NFAT2 as a Key Regulator*

Received for publication, May 23, 2002, and in revised form, August 8, 2002
Published, JBC Papers in Press, August 8, 2002, DOI 10.1074/jbc.M205063200

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To understand the molecular events coupling between cell proliferation and differentiation by elucidating genes essential for the process, we conducted a large scale gene expression analysis of an in vitro osteoclastogenesis system consisting of recombinant RANKL and mouse RAW264 cells. The entire process leading to the formation of tartrate resistant acid phosphatase-positive multinucleated cells takes 3 days and plates become fully covered with multinucleated cells at 4 days. Microarray probing at eight time points revealed 635 genes that showed greater than 2-fold differential expression for at least one time point and they could be classified into six groups by the “k-means” clustering analysis. Among a group of 106 early inducible genes (within 2–5 h after RANKL stimulation), four genes including NFAT2 were identified as genes whose enhanced expressions were fairly correlated with an efficient induction of matured osteoclasts. Moreover, cyclosporin A significantly suppressed the multinucleated cell formation accompanying the reduction of the nuclear localization of NFAT2. When the expression of NFAT2 was suppressed by introducing antisense NFAT2, multinucleated cell formation was severely hampered. Functional analysis thus combined with gene analysis by microarray technology elucidated a key role of NFAT2 in osteoclastogenesis in vitro.

Specific factors/regulatory genes playing essential roles for cellular differentiation have been identified in various systems, and they have been shown to exert their effects eventually through the induction or repression of certain groups of genes (1—4). Therefore, gene expression profiling based on fine statistical analysis in addition to an elucidation of key factors/gens might be essential to understand the molecular mechanisms underlying the differentiation process of a certain cell type. Fortunately, recent advances in the technology for assaying RNA in a highly parallel fashion (5–7), coupled with the completion/progress of several mammalian genome projects, make the approach feasible if a refined system is available. Here, we describe the broad outlines of gene expression during osteoclastogenesis in vitro, in particular during the initial stage, and explain the identification and characterization of genes essential for osteoclastogenesis on the basis of profiling characteristics. A similar approach using a different cell system was reported recently (8).

Osteoclasts are multinucleated (MN)³ giant cells and present only in bone with the capacity to resorb mineralized tissues (9). They were reported to be formed by fusion of mononuclear precursor cells derived from colony-forming unit granulocyte macrophages (CFU-GM) and branch from the monocyte-macrophage lineage during the early stage of the differentiation process (9, 10). Recently, a key factor responsible for initiating this differentiation process was identified and named receptor activator of NFκB ligand (RANKL) (or osteoclast differentiation factor (ODF)/TNF-related activation-induced cytokine TRANCE) (11–13); namely, RANKL is a type II transmembrane protein of the tumor necrosis factor (TNF) ligand family and is expressed in several cell types including activated T cells and osteoblasts. RANKL binds the receptor called receptor activator of NFκB (RANK) that is expressed in osteoclast precursors and induces osteoclast formation (14). On the basis of these findings, a system of in vitro osteoclastogenesis using the recombinant RANKL and mouse monocyte/macrophage-derived RAW264 cells was established (15–17). This system consists of a single cell type with a defined differentiation inducible factor, RANKL, and moreover, the entire process requires only 4 days to form mature osteoclasts under microscopic observation (17), making this an appropriate system to analyze a mammalian cell differentiation process.

To obtain gene expression profiles globally, RAW264 cells were cultured either in the presence or absence of RANKL, and RNA was prepared for profiling at eight time points ranging from 0 to 96 h. At each time point, RNA from RANKL-treated and -untreated cells were labeled differentially and applied to DNA microarrays to assay the relative expression levels of about 9,000 mouse genes in response to RANKL stimulation. A total of 635 genes turned out to be significantly affected by RANKL stimulation and clustering analysis of these genes made it possible to classify them into six groups. Among them, since it was reasonable to suggest that the initial stage played a crucial role in switching cells from proliferation to differentiation, we focused in the present study on the group of genes

³ The abbreviations used are: MN, multinucleated; RANK, receptor activator of NFκB; RANKL, RANK ligand; TNF, tumor necrosis factor; NFAT, nuclear factor of activated T cells; TRAP, tartrate-resistant acid phosphatase; GFP, green fluorescence protein; Tet, tetracyclin; GST, glutathione S-transferase; CsA, cyclosporin A; JNK, c-Jun amino-terminal kinase; SAPK, stress-activated protein kinase; ERK, extracellular signal-regulated kinase; NFκB, nuclear factor-κB; MKK, mitogen-activated protein kinase; MEKK1, mitogen-activated protein kinase kinase/extracellular signal-regulated kinase kinase kinase 1; TRAP, tumor necrosis factor-associated factor; IL, interleukin; RT, reverse transcriptase; AD, average difference value.

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induced within 2–5 h after RANKL stimulation. Cytokines such as IL-1α, IL-1β, and TNF-α, and a transcription factor NFAT2 were found to be included in this group. We confirmed the expression of these genes by RT-PCR and Western blotting analyses. Comparison of microarray findings during the initial stage between two different conditions in which high and low efficiency induction of tartrate-resistant acid phosphatase (TRAP)-positive MN cells were conducted, respectively, revealed the involvement of NFAT2 for MN cell formation. We further examined the behavior of NFAT2 and confirmed its crucial role in osteoclastogenesis in vitro.

MATERIALS AND METHODS

Cell Culture and Osteoclastogenesis in Vitro—Soluble recombinant RANKL was constructed and expressed as a GST fusion protein as described previously (17) and finally purified free from LPS using the Detoxi-Gel Endotoxin Removing Gel (Pierce). GST was prepared using the same procedures. RAW264 mouse monocyte/macrophage line cells (18) were obtained from the RIKEN Cell Bank (Japan) and were maintained in Eagle medium supplemented with 10% fetal calf serum and 1% non-essential amino acids (Invitrogen) as described previously (17). For osteoclastogenesis in vitro, cells were plated at a density of $1.3 \times 10^4$/cm$^2$ unless otherwise noted with 10% fetal calf serum (Hy Clone) and incubated for 24 h, and then purified RANKL was added to the final concentration of 500 ng/ml (at 0 h) when needed. Medium was changed every 3 days with Eagle medium containing RANKL and fetal calf serum as above. After culturing for the indicated periods, cells were fixed and stained for TRAP as described previously (17). When cyclopentorin A (CyA, Sigma) was administered, the indicated concentration of CyA was added and incubated for the indicated periods.

Subcellular Fractionation and Localization of NFAT2—Isolation of the nuclei and the cytosol from RAW264 cells were performed as described previously (19). Briefly, cells were harvested and treated with a hypotonic buffer (10 mM Hepes pH 7.9, 10 mM KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride and 100 units/ml aprotinin) and kept on ice for 15 min, IGEPAL CA-830 (Sigma) was added to a final concentration of 0.625%, and mixed vigorously for 10 s. Nuclei were recovered by centrifugation at 15,000 rpm for 1 min, and the resulting supernatant fraction was recovered as the cytosol. Nuclei were then treated with hypertonic buffer (10 mM Hepes pH 7.9, 400 mM NaCl, 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride and 100 units/ml aprotinin) for 15 min; the supernatant fraction was recovered as the nuclear fraction after centrifugation at 15,000 rpm for 5 min. Plasmid Construction, Transfection, and Cell Line—The pTRAP-GFP plasmid was constructed by fusing a 1.8-kb fragment containing the 5′-flanking sequence of the mouse TRAP gene (20) and pEGFP (Clontech). pTRAP-GFP was transfected into RAW264 cells with a plasmid carrying neo-resistant gene, and a clone, RAW264/TG, was isolated.

Isolation of mRNA, Reverse Transcription, and Hybridization—Pooled total RNA was isolated from RAW264 cells at the time points 0, 2, 5, 12, 24, 48, 72, and 96 h after RANKL or GST stimulation. Untreated RAW264 cells were used as 0 h control. Total RNA was purified using a Strataprep Micro and Miniprep Total RNA isolation kit (Stratagene), according to the manufacturer’s protocol. The expression of IL-1α, IL-1β, and TNF-α was examined by RT-PCR with total RNA thus prepared under the conditions described previously (21) in which a linear amplification was achieved. A pair of primers to each gene was prepared: OCTATGGCCTCATCTCATGAG and CAGTGATGTT-GGCTGCACAG for IL-1α; GGGATGATGATTGCCC and GGAATGTCCTAGAGATTCGGG for IL-1β; CCTCTCATCAGTCTAT-TGGC and CTCTCTGATCTTGGACCTC for TNF-α. For microarray probing, reverse transcription, second-strand synthesis, and probe generation were all accomplished by the standard Affymetrix protocol (Affymetrix, Santa Clara, CA) (22). Briefly, 10 μg of total RNA was used as a template for cDNA synthesis. Biotin-11-cRNA was produced using the cDNA as a template. Then the cRNA was fragmented and subjected to hybridization to oligonucleotide chips, Murine Genome U74A (Affymetrix), as described in the Gene Chip Expression Analysis Technical Manual (Affymetrix). After hybridization, bound cRNA was fluoroescently labeled using R-Phycoerythrin Streptoavidin (Molecular Probes), and the fluorescence was intensified by the antibody-amplification method. The microarrays were scanned with a laser scanner (Hewlett Packard). The derived average difference value (AD) was globally normalized and targeted to all probe sets equal to 100 before comparative analysis.

Data Analysis—To examine the expression differences of each gene, we performed comparison analyses using the Affymetrix data suite system, and the genes (probe sets) showing greater than 2-fold change in value with a difference call of 1 or D were chosen as changed genes in each comparison. Probe sets whose AD was less than 100 in all samples were excluded from further analyses due to the low confidence in the value (data not shown). The genes thus selected as changed genes, at least at one time point, were collected and each AD was transformed to a Z-score, which was calculated from the distribution of ADs of each probe set for normalization. The Z-score thus obtained was subjected to clustering analysis using the k-means method (23, 24) with Spotfire Decision Site (Spotfire Inc.). The minimum number to cover all typical patterns of expression regulation, six, was chosen by trial and error.

Antibodies, Western Blot Analysis, and Neutralization—Western blotting was carried out essentially as described previously (17). Antibodies for IL-1α, IL-1β, and TNF-α were purchased from Genzyme Techno. Anti-β-actin monoclonal antibody (AC-74, Sigma) was used. Anti-rat IgG and anti-mouse IgG were obtained from Amersham Biosciences. Anti-NFATc1 (NFAT2) monoclonal antibody (7A6, Santa Cruz Biotechnology) was used.

Expression of Antisense NFAT2 Using the Tet-Reversed Activation System—The Tet-On™ gene expression system (25) was purchased from Clonetech Inc. cDNA covering the entire coding region of the NFAT2 gene was obtained by RT-PCR using mRNA prepared from RANKL-induced RAW264 cells. The first 231 nucleotides of cDNA were inserted into the pTet-Splice in the reverse orientation, pNFAT2-AS, so that transcribed RNA could function as an antisense mRNA for NFAT2. Stable transfection of RAW264 cells with pTet-On was carried out as described previously (26), giving rise to a clone RT3. Further transfection of pASNFAT2 into RT3 gave a mixed population of stably expressed cells.

RESULTS

Efficiency and Time Course of MN Cell Formation in Vitro—Osteoclasts are giant cells, containing up to 100 nuclei, formed by fusion of mononuclear precursor cells (9). We found that the numbers of cell to be inoculated was critical for the efficiency of MN cell formation (Fig. 1A). Namely, when $1.5 \times 10^4$ and $2.5 \times 10^4$/well
104 cells were inoculated per well of 24-well plates, 2,500 MN-TRAP-positive cells were formed. However, inoculation of 104 cells resulted in 90% reduction and 104 cells gave less than 1% of the maximal number. Cell morphologies obtained after 4 days under the two conditions are shown at the bottom (Fig. 1, B and C) and importantly, cells seen in densely plated dishes were TRAP-negative. Therefore, it appeared that an inhibitory signal for the differentiation process was induced before the stage of TRAP induction, when cells were plated at the high density condition (described below).

When RAW264/TG cells in which the expression of GFP could be monitored under the control of the TRAP gene promoter were inoculated at the density of 2.5 × 104 cells per well, the expression of GFP was clearly detected at 24 h after RANKL stimulation. Cells with dendrite-like structures became apparent and significant numbers of fused cells appeared in RANKL-treated plates at 72 h. Finally, MN-TRAP-positive cells covered the plates in the RANKL-treated plates at 96 h. On the other hand, cells continued to proliferate and accumulated in GST-treated cells, and the expression of GFP was hardly detected. MN cells thus formed were shown to resorb dentine slices (data not shown; Ref. 15) and to possess hydrolyzing activity of the hydroxyapatite layer (data not shown).

### Differential Gene Expression Profiles in RANKL- and GST-treated RAW264 Cells

To understand the temporally regulated process described above at the gene level, DNA chip technology was introduced. Total RNA was prepared from time points that were chosen on the basis of previous (17) and the above observations; they were 2, 5, 12, 24, 48, 72, and 96 h after RANKL- or GST-stimulation. Total RNA thus prepared from RANKL- or GST-stimulated and the parental RAW264 cells were further purified, and labeled cRNA was prepared for DNA microarray analyses using Affymetrix oligonucleotide chips (Murine Genome U74A). A substantial number of hybridization

**Table I**

| Time points (hrs) | 2  | 5  | 12 | 24 | 48 | 72 | 96 | Total |
|------------------|----|----|----|----|----|----|----|-------|
| (A) RANKL vs untreated | 314 | 476 | 1194 | 1340 | 1297 | 1185 | 1214 | 2303 |
| (B) RANKL vs GST | 278 | 573 | 327 | 377 | 327 | 531 | 513 | 1481 |
| (C) common in (A) & (B) | 142 | 174 | 194 | 236 | 217 | 294 | 272 | 635 |

**Fig. 2.** Clustering analysis of expression profiles of 635 genes differentially expressed in osteoclastogenesis in vitro. A total of 635 genes were identified as described in the text and summarized in Table I. Z-scores were calculated from AD values of each gene and subjected to the k-means clustering analysis as described under “Materials and Methods.” The expression profiles for each gene thus obtained could be classified into six groups. A, group 1 (106 genes): genes induced during the early stage. B, group 2 (75 genes): genes induced during the middle stage. C, group 3 (153 genes): genes induced during the later stage. D, group 4 (166 genes): genes down-regulated during the early stage. E, group 5 (69 genes): genes down-regulated during the later stage; and F, group 6 (66 genes): genes unclassified. Time points examined for RANKL-stimulated cells were at 0, 2, 5, 12, 24, 48, 72, and 96 h, and those for GST-stimulated cells started at 2 h. 0 h indicates untreated RAW264 cells.
signals were determined in duplicate in independent hybridization experiments, which all showed a high degree of reproducibility. The number of probe sets (genes) that appeared to be differentially expressed by a factor greater than 2-fold between RANKL-treated and -untreated RAW264 cells at each time point are shown in the first row of Table I. In a similar fashion, genes differentially expressed (≥2.0-fold) between RANKL- and GST-treated cells at each time point were deduced and are shown in the second row. On the basis of such comparisons, genes differentially expressed in RANKL-treated cells against both GST-treated and RANKL-untreated cells could be deduced at each time point and are summarized in the third row of Table I; they consisted of 635 genes in total. We applied a non-hierarchical clustering method, k-means, to the filtered data of the 635 genes thus selected to visualize the patterns of gene expression globally, producing six major groups (Fig. 2).

**TABLE II**

| Gene list of Group I |
|----------------------|
| Gene name          | Probe set | Accession no. |
| Cytokines, Growth Factors |
| Interleukin 1 alpha | 94755_at | M14639 |
| Interleukin 1 beta | 103466_at | M15131 |
| Colony stimulating factor 3 | 94142_at | M13996 |
| MIP 2                | 101160_at | X53798 |
| Small inducible cytokine A2 | 102736_at | M19681 |
| Small inducible cytokine A3 | 102424_at | J04491 |
| Small inducible cytokine A4 | 94146_at | X50502 |
| Small inducible cytokine A5 | 98406_at | AF065947 |
| SOCS-3               | 92232_at | U88328 |
| TNF alpha            | 102629_at | D84196 |
| Cell Signaling |
| Adenosine A2b receptor | 97733_at | U05673 |
| Protein tyrosine phosphatase receptor type E | 101932_at | D83484 |
| TNF receptor member 1b | 94928_at | X87128 |
| TNF receptor member 5 | 92962_at | M83312 |
| TNF receptor member 6 | AFFX-MurFAS_at | M83649 |
| Urokinase plasminogen activator receptor | 102988_at | X62700 |
| Binder of Rho GTPase 4 | 94036_at | A1844806 |
| C-Fgr                | 94697_at | X52191 |
| C-Myc               | 104712_at | L00039 |
| DSCR 1               | 100555_at | A1846152 |
| EHD 1               | 96930_at | A1844128 |
| Hcls 1               | 99461_at | X84797 |
| Hemopoietic cell kinase | 93483_at | J03023 |
| Inhibitor of kappaB kinase epsilon | 92301_at | AB016589 |
| MARCKS-like protein | 97203_at | X61399 |
| MEK 1                | 92585_at | L05256 |
| Preoviral integration site 1 | 104533_at | AA764261 |
| Rgl 1                | 92671_f_at | U14103 |
| RGS-r               | 94578_at | U94828 |
| Rho B               | 101030_at | X99963 |
| TNF receptor-associated factor 1 | 94186_at | L35302 |
| Enzymes |
| Adenylate kinase 4 | 99959_at | AW06133 |
| Calpain 2           | 101040_at | D38117 |
| Glutaredoxin 1      | 95722_at | AB013137 |
| GTP cyclohydrolase 1 | 102313_at | L09737 |
| Manganese superoxide dismutase | 96042_at | L35528 |
| NADPH-P450 oxidoreductase | 96019_at | D17571 |
| Prostaglandin-endoperoxide synthase 2 | 104847_at | M88242 |
| Cell Surface, Extracellular |
| C-type lectin member 9 | 96551_at | AB024717 |
| Integrin alpha 5    | 103039_at | X79003 |
| PAI-1               | 94147_at | M32986 |
| Proctocadherin 7    | 102250_at | AB006758 |
| Serum amyloid A3    | 102712_at | X03505 |
| Syndecan 1          | 96033_at | Z22552 |
| Syndecan 4          | 98590_at | D89571 |
| Transporters |
| Solute carrier family 2 member 1 | 93738_at | M22998 |
| Solute carrier family 7 member 7 | 103818_at | AJ012754 |
| Solute carrier family 11 member 2 | 104451_at | A182578 |
| Solute carrier family 20 member 1 | 103065_at | M73696 |
| Transcription Regulation |
| ETS1                | 94246_at | J04103 |
| Fra-1               | 99835_at | AF017128 |
| I-kappa B alpha     | 101554_at | U57524 |
| I-kappa B beta      | 104149_at | A1642048 |
| Jun-B               | 99982_at | U19799 |
| NF-AT 2             | 102362_i_at | U20735 |
| NF-kappa-B (p105)   | 98427_s_at | M57999 |
| NF-kappa-B (p100)   | 103914_at | AW047899 |
| Rel B               | 103091_at | M83380 |
| zinc finger protein 36 | 92830_s_at | X14678 |

Role of NFAT2 in Osteoclastogenesis
The groups were named 1 through 6 based on the timing and mode of induction or suppression: Group 1 (up early); Group 2 (up middle); Group 3 (up late); Group 4 (down early); Group 5 (down late); Group 6 (others). For example, genes known as marker genes for osteoclast differentiation, such as matrix metalloproteinase 9 and vacuolar ATP synthase, were found in Group 3. In particular, genes that belonged to Group 1, which were found to be induced at 2–5 h after RANKL stimulation, were presumed to contain genes that play essential roles in determining the cell fate from proliferation to differentiation and are listed in Table II. We, therefore, focused and extended our analyses on Group 1 in the following experiments.

Osteoclast differentiation has been shown to be regulated by various cytokines and factors including RANKL secreted from osteoblasts/stromal cells, macrophage, and activated T cells (10). Among them, IL-1α, IL-1β, and TNF-α have been extensively characterized as paracrine factors (27). The present finding, therefore, that Group 1 included IL-1α, IL-1β, and TNF-α could be an important clue to understanding the physiological roles of RANKL. We further confirmed the expression levels of these genes by RT-PCR and Western blot analysis. As shown in Fig. 3A, IL-1α and IL-1β were actually found to be induced by RANKL stimulation exclusively at 2 h after stimulation, followed by rapid down-regulation, confirming the results by microarray analyses. Identical profiles were also seen at the protein level (Fig. 3B). Although the elevated expression was detected after RANKL stimulation, IL-1α was constantly detected even in control cells. On the other hand, mature forms of IL-1β and TNF-α were induced in only RANKL-stimulated cells in addition to a membrane-bound form of TNF-α (upper band in the bottom panel).

Identification of Genes Coupled with Efficient MN Cell Formation—As described above, the proceedings and efficiency of MN cell formation was greatly affected by the density of the inoculated cell numbers, and the critical time point for the cell fate appeared to be within 24 h, judging from the TRAP induction profile. It was assumed that this cell density-dependent switch might be coupled with changes in gene expression profiles and hence, we compared the gene expression levels between two conditions at 2, 5, 12, and 24 h. As a result, we could deduce four genes whose expression levels showed greater than a 2-fold difference between the two conditions even at the earliest time points (2–5 h). The four genes thus identified were Down syndrome candidate region 1 (DSCR1), nuclear factor of activated T cells 2 (NFAT2), neoplastic progression 3, and Syndecan 1. Their profiles under the two conditions are shown in Fig. 4. Among these genes, NFAT2 was different from other genes in that its expression level remained high through 24 h whereas the other three genes declined at 12 h. Moreover, only NFAT2 was a transcription factor, and its functional relation with DSCR1 was implicated (28), suggesting the possible involvement of the DSCR1/NFAT2 pathway for the MN cell formation. We then focused on NFAT2 and further characterized it in the following experiments.

NFAT was originally identified as a transcription factor responsible for the induction of IL-2 after T-cell activation and

### Table II—continued

| Gene name                        | Probe set | Accession no. |
|---------------------------------|-----------|---------------|
| **Others**                      |           |               |
| CD44 antigen                    | 103005_s_at | X66084        |
| CD83 antigen                    | 103040_at | A1837100      |
| Gadd 45b                        | 102779_at | X54149        |
| Immediate early response 3      | 94384_at | X67644        |
| Immuneresponsive gene 1         | 98774_at | L38281        |
| Mail-pending                    | 98988_at | AA614971      |
| Nef-associated factor 1         | 104755_at | AJ524778      |
| Neoplastic progression 3        | 102750_at | Z531862       |
| Nocturnin                       | 99535_at | AW047630      |
| RNA cyclase homolog             | 98923_at | A1852608      |
| Schlaen 2                       | 92471_i_at | AF099973     |
| Serine protease inhibitor 6     | 92472_f_at | AF099973     |
| **Functinally undefined genes** |           |               |
|                                 | 93702_at  | AW120888      |
|                                 | 93699_s_at | U23781       |
|                                 | 93974_at | AW212475      |
|                                 | 93975_at | A1835351      |
|                                 | 95387_f_at | AA664497     |
|                                 | 95466_at | A1837006      |
|                                 | 95637_at | A1838592      |
|                                 | 95662_at | AW211867      |
|                                 | 95673_s_at | AW124113     |
|                                 | 96206_at | A1854506      |
|                                 | 96752_at | M90551        |
|                                 | 97105_at | A642677       |
|                                 | 97252_at | AW1299265     |
|                                 | 97305_at | AW123267      |
|                                 | 98773_s_at | A1323667     |
|                                 | 99649_at | C5S552        |
|                                 | 99984_at | AA608277      |
|                                 | 102090_f_at | A1841710    |
|                                 | 102661_at | M24377        |
|                                 | 102914_s_at | U23778      |
|                                 | 103424_at | A1844439      |
|                                 | 103563_at | AW1235713     |
|                                 | 103853_at | A1845664      |
|                                 | 103891_i_at | A1197161    |
|                                 | 103892_r_at | A1197161    |
|                                 | 104396_at | AW121836      |
|                                 | 104561_at | AW120990      |
constitutes a family of four related genes: NFAT1 through NFAT4 (4, 29–31). We examined the expression profiles of NFAT1, -3, and -4 in RAW264 cells before and after RANKL stimulation. Little expression of NFAT1 and -3 were detected, whereas NFAT4 was found to be expressed constitutively throughout the conditions even in unstimulated RAW264 cells (data not shown). We, therefore, focused on NFAT2 and examined its expression profile more precisely at the protein level. As shown in Fig. 5A, the expression of NFAT2 was almost undetectable before RANKL stimulation, but the abrupt induction was detected at an approximate mass of 82 kDa on day 1. The expression level was then found to be gradually decreased during the late stage of 4-day incubation. Multiple bands were always detected and presumed to reflect the phosphorylation state (32); this was confirmed by the mobility shift of slowly migrating bands after phosphatase treatment (data not shown). The cell density-dependent expression profile of the NFAT2 protein was also confirmed by Western blotting (Fig. 5B).

Correlation of the Efficiency of MN Cell Formation and Subcellular Localization of NFAT2—The activity of NFAT2 has been shown to be regulated through the phosphorylation/dephosphorylation reaction, coupled with the change in subcellular localization. In unstimulated cells, NFAT2 is fully phosphorylated and localized in cytoplasm, and once calcineurin, a protein serine/threonine phosphatase, is activated by extracellular stimuli and following Ca2+ mediated signaling, NFAT2 forms a complex with calcineurin, resulting in the dephosphorylation of NFAT2 and translocation into nuclei to be an active form of NFAT2 (for reviews, see Refs. 31 and 33). In the present system, the induced NFAT2 protein was found as a slower migrating band and was localized exclusively in the cytoplasm on day 1. However, a faster migrating band became a major fraction and was mainly detected in the nuclear fraction on days 2 and 3 (Fig. 6). These results, therefore, suggested that a reaction causing calcineurin/NFAT2 activation took place after day 1.

Cyclosporin A (CsA), once bound to cyclophilin, has been shown to functionally associate with calcineurin and inhibit its phosphatase activity (34). It was also reported that when calcineurin is inhibited by CsA, NFAT2 protein is rapidly dephosphorylated and exported from the nucleus into the cytoplasm (35–37). Interestingly, CsA was shown to inhibit osteoclastogenesis using co-culture system (38, 39). We, therefore, made use of CsA and analyzed its effect on NFAT2 protein in addition to osteoclastogenesis in the present system. When CsA was added in the range of 10–1,000 ng/ml together with RANKL, multinuclear cell formation was significantly reduced to less than 1% of the normal condition in a dose-dependent manner (Fig. 7A). Most cells remained at the mononuclear stage by the dosage of 1,000 ng/ml of CsA and they were TRAP-positive (Fig. 7Bd). Treatment of RAW264 cells with CsA within the above range in the absence of RANKL did not affect their viability nor proliferating activity (data not shown), suggesting that the reduction of MN cell formation seen after CsA treatment was not entirely due to the effect of cell viability.

Next, we analyzed the effect of CsA on the behavior of NFAT2. Under the regular conditions, NFAT2 protein was found to become localized mainly in the nuclei at 48–72 h after RANKL stimulation (Fig. 6), at the time point when cell fusion starts to be recognized (data not shown). In contrast, when CsA was added together with RANKL, this nuclear transport was
largely suppressed in a dose-dependent manner and most NFAT2 was found in the cytoplasm when examined at 72 h (see Fig. 9). Taken together with the above notion that CsA treatment suppressed the multinuclear formation, it appears likely that NFAT2 plays a crucial role at 48–72 h, at the period after TRAP induction and before MN cell formation.

CsA and SB202190 Show Different Effects—In addition to its effect on calcineurin, CsA is also known to function on and down-regulate p38 and SAPK/JNK activities (40, 41). p38 and/or JNK/SAPK, on the other hand, were reported to play an important role in MN cell formation (16), suggesting another possibility on the inhibitory mechanism of CsA: namely, a target of CsA could be p38 and/or JNK. In that case, the effect of CsA could be mimicked by a treatment that functionally inhibits p38 and/or JNK. To clarify this possibility, we made use of a specific inhibitor, SB202190, which had been shown to be a specific inhibitor of p38 at low concentrations (4 μM) and was also reported to inhibit JNK at higher concentrations (40 μM) (42, 43). When SB202190 was administered at 10–40 μM with RANKL, multinucleated cells were hardly detected and mononuclear cells without TRAP activity were observed as reported previously (16). In contrast, when CsA suppressed the multinuclear cell formation, the mononuclear cells observed were TRAP-positive (Fig. 8B, d and e). Therefore, the effect of CsA appeared to be different from that of SB202190 and CsA was presumed to suppress multinuclear formation not through the JNK/p38 pathway.

Extracellular signal-regulated kinases (ERKs) are comprised of MAP kinase superfamily with p38 and JNK/SAPK (44). All these kinases were reported to physically associate with the amino-terminal regulatory domain of NFAT2 and can directly phosphorylate functionally important residues including Ser-172 (45). In contrast to SB202190, PD98059, which is an inhibitor of MEK1, showed no inhibitory effect on RANKL-induced differentiation (data not shown), confirming the previous findings of Matsumoto et al. (16).

Suppression of NFAT2 Hampered MN Cell Formation—To examine the role of NFAT2 on the differentiation process more directly, we introduced antisense NFAT2 expression plasmid into RAW264 cells whose expression was regulated by the Tet reversed activation system (25). We used mixed populations of stably expressed cells for that purpose. The expression level of NFAT2 protein was found to be lower than that of the parental RT3 cells even in the absence of an inducer, doxycycline, suggesting that the regulation of the pTet-Splice was leaky and the...
message of antisense NFAT2 was expressed constitutively (Fig. 9A). However, when MN cell formation was examined using 20 μM doxycycline in a mixed population, MN cells were hardly detected compared with either wild-type or the RT3 cells (Fig. 9, B and C) at 72 h after RANKL stimulation. These results further confirmed the above findings that NFAT2 plays a key role in the process of MN cell formation in RANKL-stimulated RAW264 cells.

DISCUSSION

In our previous study using the same system described in the present study, we analyzed the expression profiles of cell cycle regulatory genes during the initial phase in committed cells to clarify the mechanisms controlling the coupling between withdrawal from the cell cycle and differentiation. As a result, we found that RANKL could induce pleiotropic effects on cell cycle regulatory genes such as Rb, p21, CycD3, and cdk6 (17). In the present study, we conducted a more systematic and a large scale gene expression analysis on the entire process of osteoclastogenesis in vitro, and identified 635 genes to be differentially regulated at least at one time point. Clustering of these genes made it possible to classify them into six groups and such analysis revealed temporally regulated gene expression profiles during the RANKL-induced differentiation process, providing a basis for understanding osteoclastogenesis at the gene level, in conjunction with observations at the cellular level.

In the present system consisting of RAW264 cells and the soluble form of recombinant RANKL, RANKL binds to its receptor, RANK, and RANK in turn associates with tumor necrosis factor receptor (TNFR)-associated factor (TRAF) family proteins inside the cell (reviewed in Ref. 46). Among the TRAF family of proteins, TRAP2, TRAP5, and TRAP6 appeared to activate the downstream molecules such as NFκB and JNK (47). In this regard, TRAP6 has been shown to activate M KK2/4/6/7 (48) and MEKK1 (49). In particular, gene targeting of TRAP6 impaired the bone resorbing activity of osteoclasts, suggesting that TRAP6 plays a crucial role in the RANKL/RANK signaling pathway in inducing commitment to differentiation (50–52). IFN-γ signaling has been shown to suppress the differentiation potential of osteoclast precursor cells by destabilizing TRAP6 (53). We identified the suppressor of the cytokine signaling 3 (SOCS3) gene in Group 1; SOCS family is known to function as a physiological suppressor of cytokine signaling through JAK/STAT (54, 55). RANKL, therefore, appears to induce factor(s) capable of stabilizing the TRAF family, in addition to direct activation of the TRAF family.

To identify functionally relevant genes for osteoclastogenesis, we made use of our findings that cell densities upon RANKL stimulation significantly affected the efficiency of MN cell formation. Comparison of microarray findings obtained at the very early stage under the two different conditions, one induces efficient MN cell formation and the other results in weak induction, could deduce four genes including DSCR1 and NFAT2 as candidate genes. Since DSCR1 was reported to be an inhibitor of calcineurin (28, 56), which has been presumed to be an activator of NFAT, this finding highlighted a signaling pathway involving NFAT2 as an important factor for osteoclastogenesis. With regard to this, DSCR1 was found to express
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