is a secreted TNF receptor–related protein, which was isolated as a protein-inhibiting bone resorption.[9,10] Because OPG does not have a transmembrane domain, it works as a decoy receptor and inhibits bone resorption.[7,8] Osteoclastogenesis can be reproduced efficiently by coculture in vitro using bone marrow stromal cells and bone marrow macrophages or spleen cells.[11–14] The essential role of bone marrow stromal cells in in vitro osteoclastogenesis is explained by the fact that RANKL is expressed in the bone marrow stromal cells on stimulation with 1α,25-dihydroxyvitamin D_3 and dexamethasone.[3,7] Actually, bone marrow macrophages or spleen cells differentiated into osteoclasts without stromal cells when the soluble form of recombinant RANKL protein was added to the culture medium.[2,3] The essential role of the RANKL/RANK system in osteoclastogenesis was confirmed in mice with a disrupted *opgl* gene. The mice showed severe osteopetrosis and a defect of tooth eruption caused by complete lack of osteoclasts.[15]

Bone metabolism must be regulated strictly to maintain bone structures, to heal fractures, and to supply calcium ion. We showed dynamic change in the biological activity of bone cells under physiological and pathological conditions.[16,17] Physiologically, bone formation and bone resorption are synchronized nicely, with an imbalance leading to osteopetrosis or osteoporosis. A decrease in bone volume occurs in aged men.[18] In the aged male rat bones, most osteoblasts looked flat-shaped and expressed much less type I collagen and osteocalcin messenger RNA (mRNA) than the cuboidal osteoblasts in the young rat bones.[16] We also found that osteopontin mRNA was dominantly expressed in osteoclast progenitor-like mononuclear cells and osteoclasts rather than osteoblastic cells,[16,17,19] and the expression in these bone resorbing cells greatly decreased in the aged rat bones.[16] These findings indicate that the bone metabolism in aged rat bones is less active than that in young rat bones and support that osteoporosis in aged men occurs because of an imbalance between bone resorption and bone formation at low turnover of bone. Postmenopausal osteoporosis is thought to be caused by estrogen deficiency and is reproduced in an animal model by ovariectomy.[20] Overproduction and overactivation of osteoclasts have been observed in bones of ovariectomized rats.[17,21–25] Previously, we showed that both the expression of type I collagen and osteocalcin in osteoblasts and the expression of osteopontin in bone-resorbing cells increased in the ovariectomized rat bones.[17] These data confirmed that postmenopausal osteoporosis was caused by an imbalance of bone resorption and bone formation under conditions of increased bone turnover. The importance of the RANKL/RANK system for osteoclastogenesis strongly suggests that the system is associated with the pathogenesis of age-dependent reduction in bone volume and postmenopausal osteoporosis.

In this study, we analyzed the expression of mRNA for RANKL, RANK, and OPG in bones of 8-week-old and 2.5-year-old male rats and sham-operated and ovariectomized female rats. Relatively constant expression levels of RANKL and RANK mRNA in the endosteal cells in the aged rat bones indicated a different regulatory mechanism for the expression of RANKL and RANK mRNA from that of bone matrix proteins, which was analyzed previously.[16] On the other hand, the expression of RANKL, RANK, and OPG mRNA increased in the ovariectomized rat bones like the expression of bone matrix proteins, which was reported previously.[17] These results strongly suggested that the RANKL/RANK/OPG system was closely involved in the pathogenesis of bone loss by aging and estrogen deficiency.

MATERIALS AND METHODS

Animals and tissue preparation

Eight-week-old male and 10-week-old female Wistar rats were purchased from SRL (Shizuoka, Japan). Male Wistar rats (2.5 years old) were supplied from SPF Aging Farm of Tokyo Metropolitan Institute of Gerontology (Tokyo, Japan). The male Wistar rats from the farm, which were purchased from SRL, were kept from the time they were 4 weeks old, and mean survival of the 2.5-year-old male Wistar rats was 8.8% (Department of Laboratory Animal Science, Tokyo Metropolitan Institute of Gerontology, unpublished data, 1998). From the farm, four healthy 2.5-year-old male rats were supplied for experiments. The 8-week-old and the 2.5-year-old male rats were anesthetized with ether and killed by cardiac perfusion. The 10-week-old female Wistar rats were anesthetized and bilaterally ovariectomized or sham-operated. These animals were killed by cardiac perfusion 3 weeks after the operation. The distal portion of the femur was dissected from each animal and fixed for 24 h at 4°C in 4% formaldehyde in 0.1 M phosphate buffer (pH 7.2), which was prepared just before use. All fixed samples were decalcified with 0.5 M EDTA solution at 4°C for up to 1 month. The decalcified samples were dehydrated in ethanol, cleared in chloroform, and embedded in paraffin, and then 4-μm thick sections were made. Four animals for each group were used for the experiments.

Preparation of RNA probes

Complementary DNA (cDNA) fragments of mouse RANKL and RANK were cloned from mouse thymic cDNA, and cDNA fragments of mouse OPG were cloned from mouse bone marrow macrophage cDNA by reverse-transcription–polymerase chain reaction (RT-PCR) method. The RT-PCR was carried out with 40 cycles of denaturation at 98°C for 20 s and annealing and extension at 68°C for 2 minutes using LA *Taq* polymerase (Takara Co., Shiga, Japan) and the following primers:

- RANKL. RANKL5, 5′-ATGAAACAAGCCTTTCAG-GGGGCCGTGCA-3′; and RANKL3, 5′-TCAGTCT-ATGTCCTGAACTTTGAAAGCCCC-3′
- RANK. RANK5, 5′-TCACCGGGACTGAAAGCAC-GGTGG-3′; and RANK3, 5′-TGGGCTCCATCA-CCATGCCAGCAG-3′
- OPG. OPG5, 5′-GGACAGTTTGCCTGGGACCAA-AGTGAATGC-3′; and OPG3, 5′-TGAAGCTGTGC-AGGAACCTCATGGTCTTCC-3′

The cDNA fragments were cloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA). The vectors including

the cDNA fragment for RANKL, RANK, or OPG were linearized and transcribed using T7 RNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany) to generate uridine 5'-triphosphate (UTP)-labeled single-stranded antisense and sense RNA probes, respectively. The [^{33}P]-labeled probes were used for hybridization at a concentration of 1×10^7 cpm/ml in 50% formamide, 10% dextran sulfate, 0.6 M NaCl, 10 mM Tris-HCl (pH 7.6), 1 mM dithiothreitol (DTT), and 0.2 μg/ml of yeast total RNA.

In situ hybridization

Treatment of the slides and hybridization conditions were as described previously.[19] Briefly, after removal of the paraffin with xylene, the sections were treated with 2 μg/ml of proteinase K (Sigma Chemical Co., St. Louis, MO, USA) at 37°C for 15 minutes, fixed for 15 minutes with the same fixatives as described previously, treated with 0.25% acetic anhydride in 0.1 M triethanol amine-HCl (pH 8.0) for 10 minutes, dehydrated in ethanol, and air-dried. The hybridization solution was spread over the sections and the slides were incubated overnight at 60°C in plastic humidified boxes. After hybridization, the sections were washed twice in 50% formamide solution containing 2× SSC and treated with 12.5 μg/ml of RNAse A (Sigma Chemical Co.). The sections were then washed twice in 2× SSC and twice in 0.2× SSC at 60°C for 30 minutes each time, dehydrated in ethanol, and finally dipped in NTB-3 emulsion (Eastman Kodak, Rochester, NY, USA) diluted 1:1 with a 2% glycerol solution. The dipped slides were exposed at 4°C for 3 weeks. The exposed slides were developed in D-19 developer, fixed in F-5 fixative, and finally counterstained with hematoxylin and eosin.

TABLE 1. SUMMARY OF PROFILES OF THE EXPRESSION OF RANKL, RANK, AND OPG MRNA

	8 w	*2.5 y*	*sham*	*ovx*
RANKL				
Cortical periosteum[a]	++	+	+	+
Cortical endosteum[a]	+	+	+	+
Trabecular endosteum[b]	−	−	−	+
Growth plate hypertrophic chondrocytes	+	−	+	++
Osteocytes	−	−	−	−
RANK				
Cortical periosteum[a]	+++	+	++	++
Cortical endosteum[a]	++	+	+	+
Trabecular endosteum[c]	+	+	+	++
Growth plate	−	−	−	−
Osteocytes	−	−	−	−
OPG				
Cortical periosteum[a]	+	−	−	−
Trabecular endosteum[c]	−	−	−	+
Growth plate proliferative chondrocytes	+	+	−	−
Growth plate resting chondrocytes	−	−	−	+
Articular proliferative chondrocytes	+	−	+	+
Articular resting chondrocytes	−	−	−	+++
Osteocytes	+	−	+	++

8w, 8-week-old rat; 2.5 y, 2.5-year-old rat; sham, sham-operated rat; OVX, ovariectomized rat.
[a] Metaphyseal region.
[b] Primary spongiosa.
[c] Primary and secondary spongiosas.

RESULTS

The expression profiles of RANKL, RANK, and OPG mRNA were summarized in Table 1. The grading of the expression level represents total amount of the expression in each region. Profiling of the sites and cells that appear in the table were shown in Fig. 1.

The expression of RANKL mRNA

The expression levels of RANKL mRNA were compared between the femurs of 8-week-old and 2.5-year-old male rats. In the 8-week-old rat bones, RANKL mRNA was detected most prominently in a subset of periosteal cells of the metaphyseal cortical bone (Fig. 2A, arrows). Lower levels of expression were detected in a subset of endosteal cells (Fig. 2A, arrowheads). In addition, RANKL mRNA was detected in hypertrophic chondrocytes of the growth plate (Fig. 2C, arrows). In the primary spongiosa, RANKL mRNA was not detected clearly (Fig. 2C). In the 2.5-year-old rat bones, RANKL mRNA was detected in a subset of periosteal (Fig. 2B, an arrow) and endosteal cells (Fig. 2B, arrowheads) of the metaphyseal cortical bones. The expression profile in the 2.5-year-old rat bones was similar to that in the 8-week-old rat bones except for the periosteal region. In the periosteal region, cells with a positive signal were fewer than in the 8-week-old rat bones although the expression level in each cell was similar to the 8-week-old rat bones (Figs. 2A and 2B, arrows). In the 2.5-year-old rat bones, the growth plate was composed of several layers of resting and/or proliferative chondrocytes, and hypertrophic chondrocytes were not present. In this region, little RANKL mRNA was expressed (Fig. 2D).

Next, the expression of RANKL mRNA was analyzed in the femurs of sham-operated and ovariectomized female rats. Ten-week-old female rats were sham-operated or ovariectomized and killed 3 weeks after each operation. The expression of RANKL mRNA was detected in a subset of periosteal (Figs. 2E and 2F, arrows) and endosteal (Fig. 2F, arrowheads) cells of the sham-operated and ovariectomized rat bones. The expression level was similar in this region between the sham-operated and ovariectomized rat bones (Figs. 2E and 2F). In the growth plate of the sham-operated rat bones, RANKL mRNA was detected in the hypertrophic chondrocytes (Fig. 2G, arrows). In the ovariectomized rat bones, both the number of cells expressing RANKL mRNA and the expression level of RANKL mRNA in each cell increased in comparison to the sham-operated rat bones (Fig. 2H, arrows). In addition to being up-regulated in the hypertrophic chondrocytes, the expression of RANKL

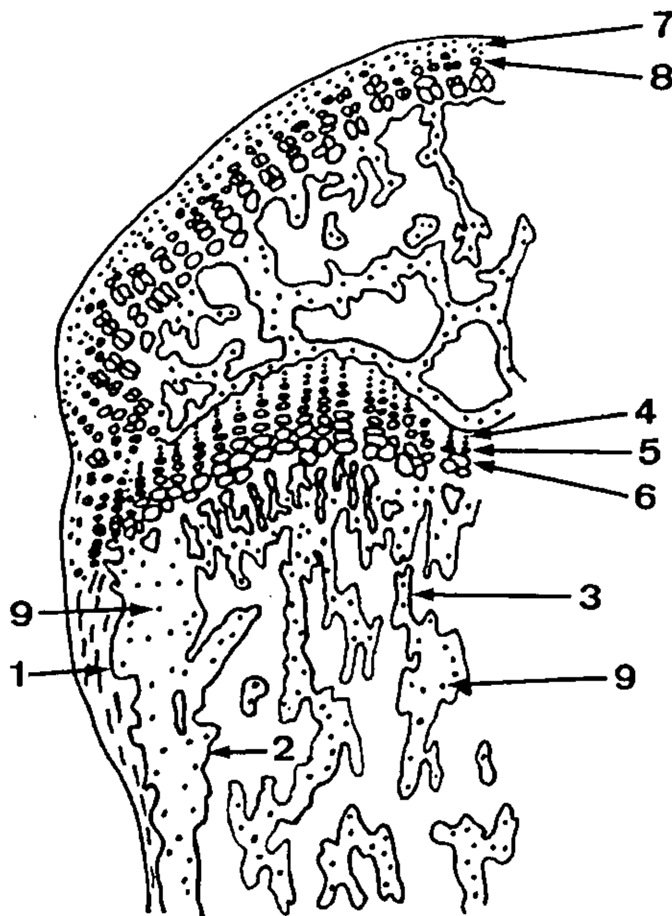

FIG. 1. Profiling of the sites and cells in the femur analyzed in this study. 1, cortical periosteum; 2, cortical endosteum; 3, trabecular endosteum; 4, growth plate resting chondrocyte; 5, growth plate proliferative chondrocyte; 6, growth plate hypertrophic chondrocyte; 7, articular resting chondrocyte; 8, articular proliferative chondrocyte; 9, osteocyte.

mRNA was induced in a subset of cells in the primary spongiosa of the ovariectomized rat bones (Fig. 2H, arrowheads). In the primary spongiosa of the sham-operated rat bones, RANKL mRNA was not clearly detected (Fig. 2G).

The expression of RANK mRNA

In the metaphyseal cortical bone of the 8-week-old rats, high levels of expression of RANK mRNA were detected in relatively small cells (Fig. 3A, arrows) and in large cells (Fig. 3A, arrowheads) in the periosteum and endosteum. In the periosteum of the 2.5-year-old rat bones, both the number of cells expressing RANK mRNA and the expression level in each cell were less than those in the 8-week-old rat bones, but in the endosteum of the 2.5-year-old rat bones, the number of cells expressing RANK mRNA changed little and only the level of the expression of RANK mRNA decreased in comparison to the 8-week-old rat bones (Figs. 3B and 3C). In the 2.5-year-old rat bones, RANK mRNA was detected in mononuclear cells (Fig. 3C, arrows) and multinuclear cells (Figs. 3B and 3C, arrowheads). The expression profile in the trabecular bones of each animal was similar to that in the metaphyseal cortical bones (data not shown).

In the sham-operated and ovariectomized rat bones, RANK mRNA was detected in small (Figs. 3D and 3E, arrows) and large (Figs. 3D and 3E, arrowheads) cells in the periosteum and endosteum of the metaphyseal trabecular bones. The level of expression in these regions was similar between sham-operated and ovariectomized rat bones. However, in the trabecular bones, the expression in each cell was stronger in the ovariectomized rats than in the sham-operated rats (Figs. 3F and 3G).

The expression of OPG mRNA

OPG mRNA was detected in the cartilage rather than in the bone. In the 8-week-old rat bones, relatively strong expression of OPG mRNA was detected in the proliferative chondrocytes of the articular cartilage (Fig. 4A, arrows). In addition, the mRNA also was detected in some proliferative chondrocytes of the growth plate (Fig. 4C, arrows). In the 2.5-year-old rat bones, OPG mRNA also was detected in the proliferative chondrocytes of the atrophic growth plate (Fig. 4D, arrows), but the number of cells expressing OPG mRNA and the expression level in each cell were similar to those in the 8-week-old rat bones. In the articular cartilage, the expression was undetectable (Fig. 4B). Outside of the cartilage region in the 8-week-old rats, OPG mRNA was detected in a subset of cells in the periosteum of the metaphyseal cortical bones (Fig. 4E, arrows). In this region, the mRNA also was detected in a subset of osteocytes (Fig. 4E, an arrowhead). In the 2.5-year-old rat bones, the expression was not detected clearly in the metaphyseal cortical bone region (Fig. 4F). In the trabecular bones of the 8-week-old and 2.5-year-old rats, little OPG mRNA was expressed (data not shown).

In the articular region of the sham-operated rat bones, OPG mRNA was detected in the proliferative chondrocytic cells (Fig. 5A, arrowheads). In addition, the mRNA was detected in some osteocytes in the metaphyseal and diaphyseal cortical bones (Fig. 5C, arrowheads). In the region of the growth plate, little OPG mRNA was seen in the sham-operated rats (Fig. 5E) in contrast to 8-week-old and 2.5-year-old male rats (Figs. 4C and 4D). In the sham-operated rats, the expression also was weak in the trabecular bone region (Fig. 5G). Three-weeks after the ovariectomy, the expression of OPG mRNA greatly increased in some regions of the bone. Prominent expression was detected in resting chondrocytes of the articular cartilage (Fig. 5B, arrows). In this region, the mRNA also was detected in some proliferative chondrocytes (Fig. 5B, arrowheads). In the cortical and trabecular bones, both the number of osteocytes expressing OPG mRNA and the expression level of OPG mRNA in each cell increased (Fig. 5D, arrowheads). In the growth plate region, weak expression was detected in some resting chondrocytes (Fig. 5F, arrows). In addition, many cells expressing OPG mRNA were seen in the primary (Fig. 5H) and secondary spongiosa (data not shown) of the ovariectomized rat bones.

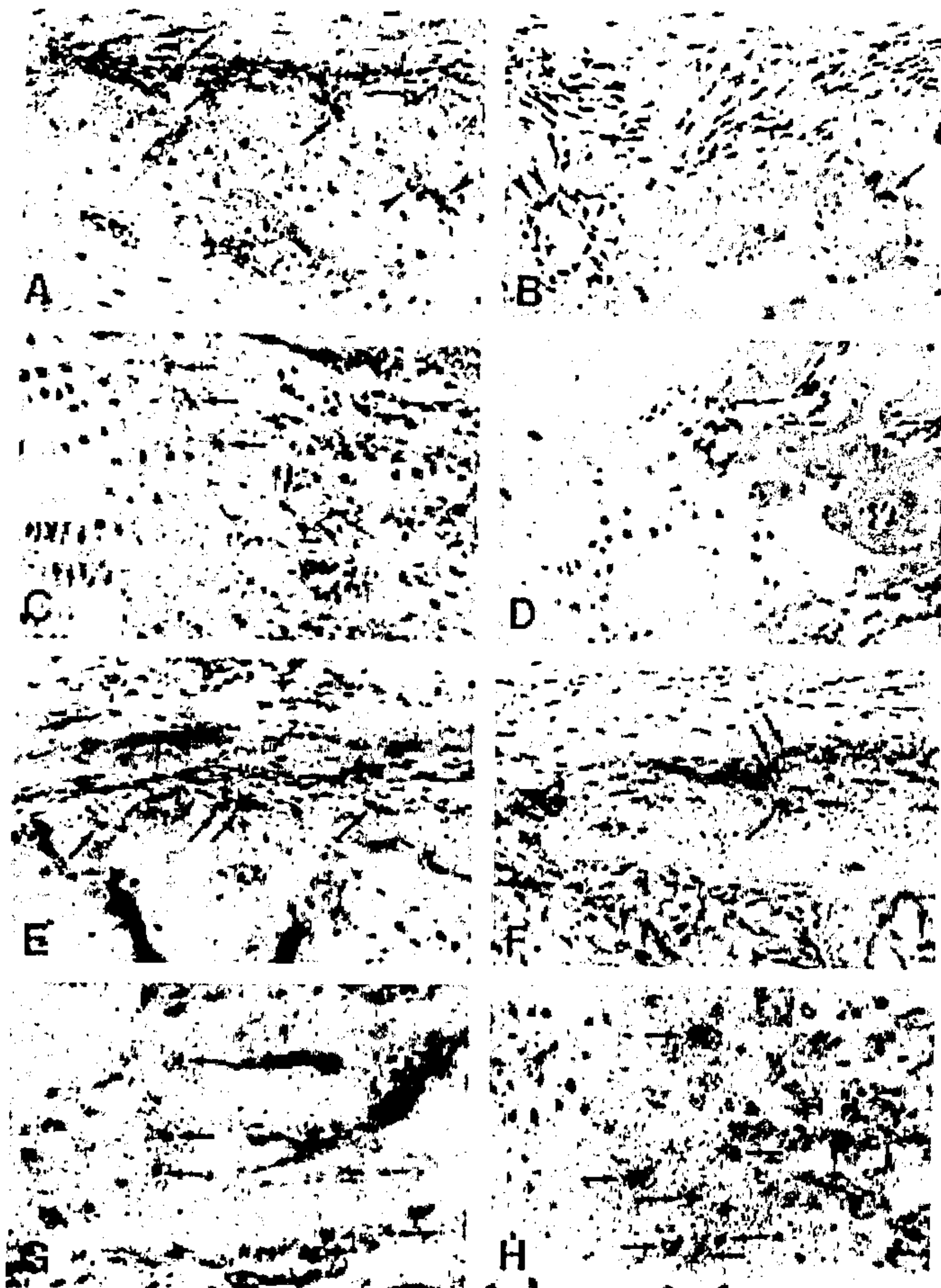

FIG. 2. The expression of RANKL mRNA in the femurs of (A and C) 8-week-old male, (B and D) 2.5-year-old male, (E and G) sham-operated female, (F and H) and ovariectomized rats. (A and B) The expression in the periosteal (arrows) and endosteal (arrowheads) cells of the metaphyseal cortical bones. (C) The expression in the hypertrophic chondrocytes (arrows) of the developing growth plate. (D) No expression of RANKL mRNA in the atrophic growth plate region of the 2.5-year-old rat bone. (E and F) The expression in the metaphyseal cortical bone region of the sham-operated and ovariectomized rats. Arrows indicate the expression in the periosteum and arrowheads indicate the expression in the endosteum. (G and H) The expression in hypertrophic chondrocytes of the growth plate (arrows). In the ovariectomized rat bone, strong expression in the hypertrophic chondrocytes (panel H, arrows) and induction of RANKL mRNA in the cells on the trabecular bones (panel H, arrowheads) are seen (×300).

For all these experiments, sense control hybridization was performed and no specific signal was detected (data not shown).

DISCUSSION

In the 8-week-old rat bones, strong expression of RANKL and RANK mRNA was detected in the periosteum and endosteum of the metaphyseal cortical bones. In the 2.5-year-old rat bones, the expression of RANKL and RANK mRNA in the periosteum greatly decreased, but the expression in the endosteum was relatively stable. Metaphyseal cortical bone is the region where bone reconstruction actively occurs during bone development.[29] Therefore, the high level of expression of RANKL and RANK mRNA in the epiphyseal cortical bone of the 8-week-old rats was thought to be induced by active reconstruction in the developing bone. In addition, the decreased expression of RANKL mRNA in the growth plate of the 2.5-year-old rat bones might be caused by a less active bone metabolism than that in the 8-week-old rat bones. Except for the regions where bone metabolism was influenced greatly by the growth of bone, the difference in the expression level of RANKL and RANK mRNA between young and old rat

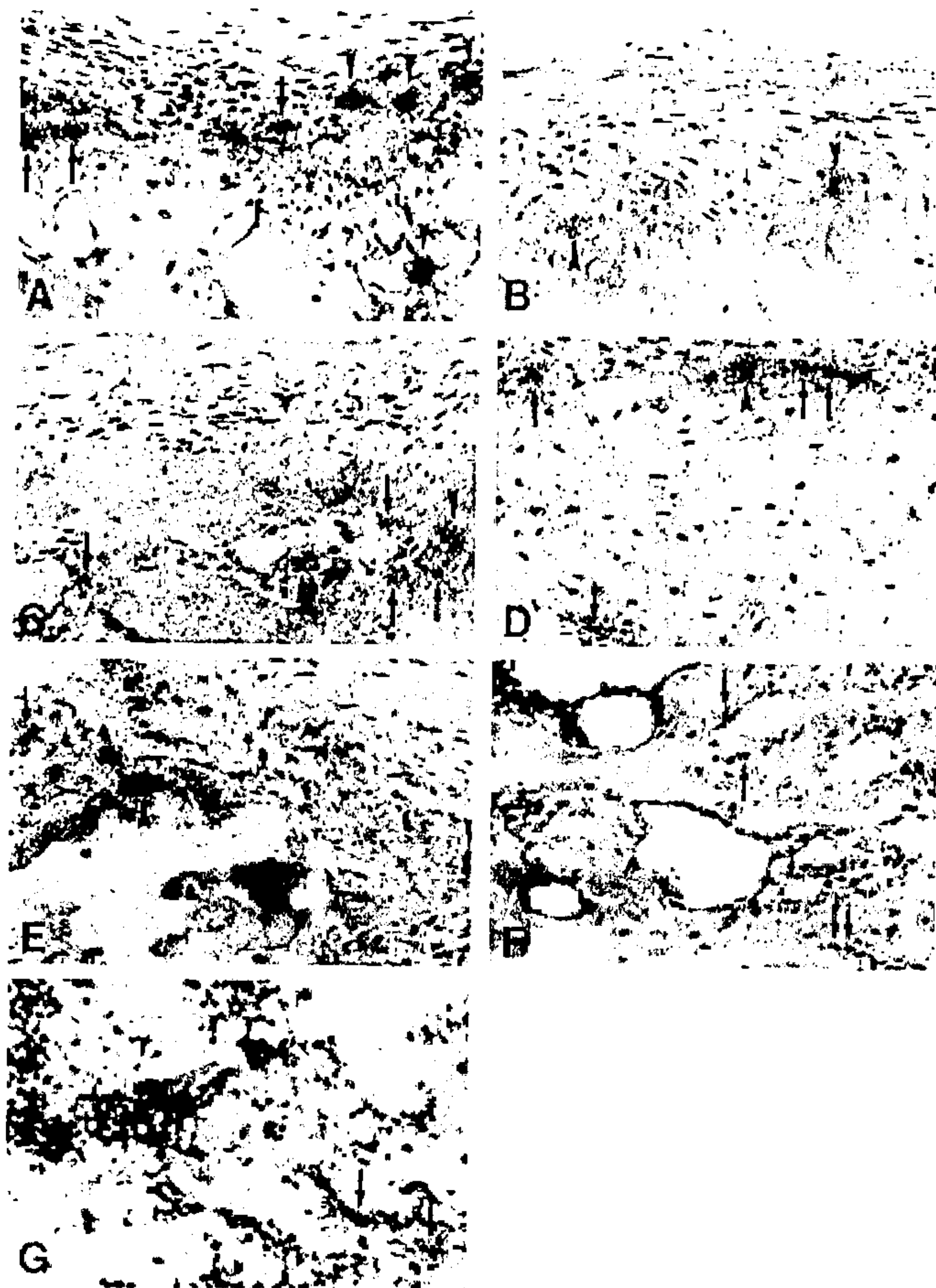

FIG. 3. The expression of RANK mRNA in the femurs of (A) 8-week-old male, (B and C) 2.5-year-old male, (D and F) sham-operated female, and (E and G) ovariectomized rats. (A) Strong expression of RANK mRNA in the small (arrows) and large (arrowheads) cells in the metaphyseal cortical bone of the 8-week-old rat. (B and C) The expression in the osteoclast-like cells (arrowheads) and mononuclear cells (arrows) in the periosteum (B) or endosteum (C) of the metaphyseal cortical bone of the 2.5-year-old rat. (D and E) The expression in the metaphyseal cortical bones of the sham-operated (D) and ovariectomized (E) rats. Signals in the small (arrows) and large (arrowheads) cells are seen in both specimens. (F and G) The expression of RANK mRNA in cells on the surface of the trabecular bones (arrows). (G) Strong expression is seen in the ovariectomized rat bone (×300).

bones was not great. Considering the marked decrease in the expression of mRNA for bone matrix proteins in aged rat bones,[16] the decrease in bone resorption may be milder than that of bone formation in the aged rat bones. This may explain the pathogenesis of osteoporosis in aged men.

We previously reported that the expression of bone matrix proteins increased in ovariectomized rat bones in addition to the number and activity of osteoclasts.[17] The data indicated that osteopenia in ovariectomized rat bones was induced by an imbalance of bone resorption and bone formation under conditions of high turnover of bone. The RANKL/RANK system, a key regulator of osteoclastogenesis, was expected to be stimulated in the ovariectomized rat bones. Actually, in the ovariectomized rat bones, an increase in the expression of RANKL mRNA was seen in the hypertrophic chondrocytes of the growth plate, and induction of the expression was seen in the cells of the primary spongiosa (Figs. 2G and 2H). An increase in the expression of RANK mRNA was seen in the cells of the trabecular bones (Figs. 3F sand 3G). However, in the cortical bone region, the expression profiles of RANKL and RANK mRNA changed little between sham-operated and ovariectomized rat bones (Figs. 2E, 2F, 3D, and 3E). In the ovariectomized rats, bone volume decreased more prominently in the trabecular bones than in the cortical bones, at least up to 6 weeks after the operation.[17] Therefore, an increase in the

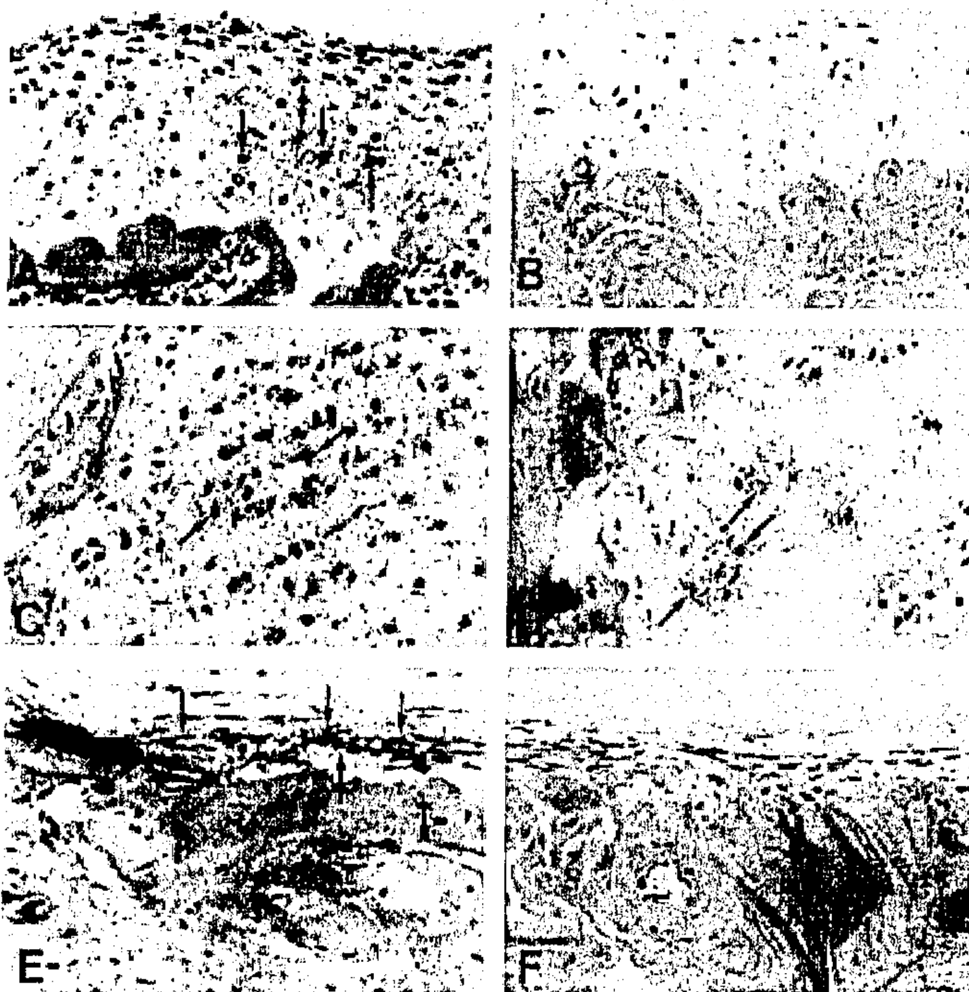

FIG. 4. The expression of OPG mRNA in the femurs of (A, C, and E) 8-week-old male and (B, D, and F) 2.5-year-old male rats. (A) The expression in proliferative chondrocytes in the articular region of the 8-week-old rat (arrows). (B) No expression of OPG mRNA in the articular region of the 2.5-year-old rat. (C and D) The expression in the proliferative chondrocytes in the growth plate of the (C, arrows) 8-week-old and (D, arrows) 2.5-year-old rat bones. (E) The expression of OPG mRNA in a subset of periosteal cells (arrows) and an osteocyte (an arrowhead) of the metaphyseal cortical bone of the 8-week-old rat. (F) Equivalent region to panel E in the 2.5-year-old rat. No expression of OPG mRNA in this region (×300).

expression of RANKL and RANK mRNA in the trabecular bones rather than in the cortical bones was thought to explain why the increase in bone resorption caused by estrogen deficiency was more severe in the trabecular bones than in the cortical bones.[17] The mechanism of the differential regulation of the expression of RANKL and RANK mRNA in different portions of the bone was not clear, but because the ovariectomy induced systemic hormonal change, the differential response of trabecular bone cells and cortical bone cells to estrogen deficiency might be caused by the difference of cell populations between the trabecular bone region and cortical bone region. In addition, increased expression of RANKL mRNA in the hypertrophic chondrocytes of the ovariectomized rat bones suggests that abnormally excessive bone resorption occurs just after endochondral bone formation in the region.

Also, it was interesting that the expression level of RANK mRNA was high in osteoclastic cells and osteoclast progenitor-like mononuclear cells on the surface of the cortical and trabecular bones but not in the bone marrow cells. Our PCR analyses indicated that bone marrow macrophages express RANK mRNA (Ikeda and Utsuyama, unpublished data, 2000), and the expression was thought to be enhanced on the surface of the cortical and trabecular bones. These results suggest that the expression of RANK mRNA increases to a level high enough to induce osteoclastogenesis on the surface of the bone matrix, and this might explain why osteoclasts are formed only on the surface of the bone matrix.

OPG mRNA was shown to be expressed in cartilages in a mouse embryo,[9] but the expression profile in mature bone tissue had not been reported. In this study, we showed that OPG mRNA was expressed in proliferative chondrocytes, osteocytes, and a subset of periosteal cells in the 8-week-old rat bones. Relatively strong expression was seen in the proliferative chondrocytes and osteocytes. OPG works as a decoy receptor for RANKL and inhibits osteoclastogenesis by competing with RANK when binding to RANKL.[8] The expression profile of OPG mRNA was different from that of RANK mRNA. The expression of OPG mRNA in the proliferative chondrocytes may not affect osteoclastogenesis considering the fact that there is no vascularization in the cartilage, but there is a possibility that OPG is accumulated in the cartilage and protects it from resorption. Surprisingly, in the ovariectomized rat bones, strong expression of OPG mRNA was induced in the resting chondrocytes (Fig. 5B). In human osteoblastic cells, the expression of OPG mRNA was indicated to be stimulated

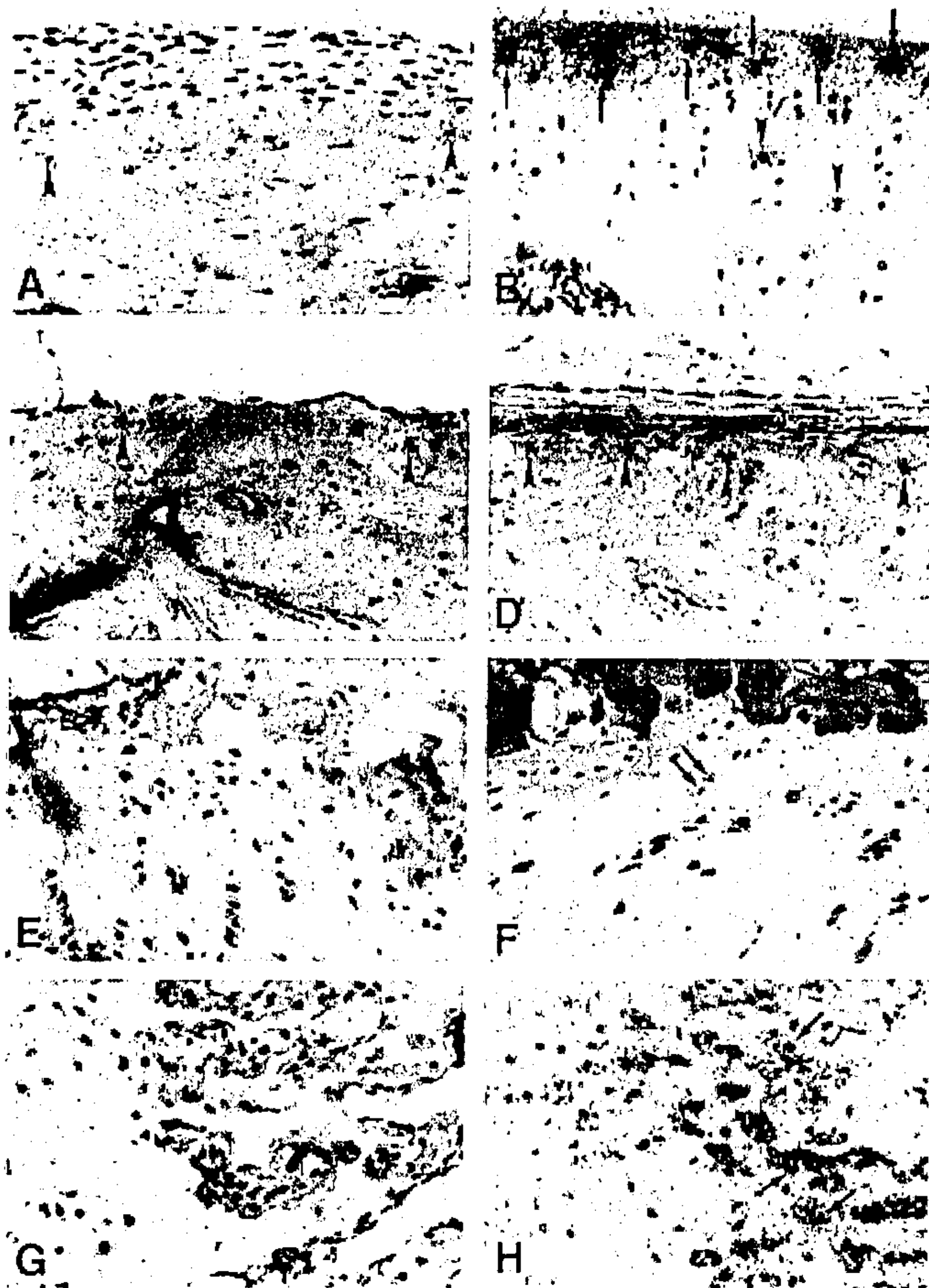

FIG. 5. The expression of OPG mRNA in the femurs of (A, C, E, and G) sham-operated female and (B, D, F, and H) ovariectomized rats. (A and B) The expression of OPG mRNA in the articular region. Proliferative chondrocytes with positive signal (arrowheads) are seen in both (A) sham-operated and (B) ovariectomized rat bones. Note the high level of expression in the resting chondrocytes in the ovariectomized rat bone shown in panel B (arrows). (C and D) The expression of OPG mRNA in the osteocytes (arrowheads) of the cortical bones in the (C) sham-operated and (D) ovariectomized rats. An increase in the number of osteocytes with a positive signal is observed in the ovariectomized rat bone shown in panel D. (E and F) Upper portion of the growth plate and a part of the epiphysis. (E) No expression of OPG mRNA in the sham-operated rat bone and (F) weak expression in the resting chondrocytes (arrows) in the ovariectomized rat bone. (G and H) Lower portion of the growth plate and a part of the primary spongiosa of the metaphysis. (G) No expression of OPG mRNA in the sham-operated rat bone (H) and mRNA expression in a subset of cells on the surface of the primary spongiosa (arrows) in the ovariectomized rat bone (×300).

by estrogen.[27,28] In addition, serum OPG was shown to increase in women with postmenopausal osteoporosis.[29] Our data seemed to conflict with these data. In this study, we could not detect the expression of OPG mRNA in osteoblastic cells, and in vivo change in expression of OPG mRNA in osteoblasts remained unknown. The meaning of this strong induction of OPG mRNA in the resting chondrocytes and osteocytes was not clear, but our results strongly suggested that regulation of OPG expression in chondrocytes and osteocytes was different from that of osteoblasts.

The expression of OPG mRNA in the osteocytes is interesting. The increase in the number of osteocytes expressing OPG mRNA in the ovariectomized rat bones suggests a protective reaction against excess bone resorption caused by ovariectomy. The excess bone resorption exposes the osteocytes expressing OPG mRNA at the surface of the bone, which may inhibit osteoclastogenesis. In the ovariectomized rat bones, the expression of OPG mRNA also was induced in cells on the surface of the trabecular bones (Fig. 5H). In this study, we could not characterize these cells, but there was a possibility that they were identical to the osteocytes expressing OPG mRNA, which were exposed at the surface of the bone.

There were some inconsistencies in the expression between 8-week-old male rat bones and sham-operated female

rat bones. There are three possibilities to explain these inconsistencies: difference between 8-week-old rat bones and 13-week-old rat bones, difference between nontreated rat bones and sham-operated rat bones, or difference between male rat bones and female rat bones. In the proliferative chondrocytes, OPG mRNA was detected only in the male rat bones and was not detected in the female rat bones irrespective of age and operation. Therefore, it was suggested that the difference in the expression of OPG mRNA in the proliferative chondrocytes was caused by sex difference.

In this study, we analyzed profiles of the expression of RANKL, RANK, and OPG mRNA in the 8-week-old and 2.5-year-old male rat bones and in the sham-operated and ovariectomized female rat bones. The expression of RANKL, RANK, and OPG mRNA was relatively stably maintained in the endosteal region. In addition, high expression of OPG mRNA was induced in resting chondrocytes and osteocytes in the ovariectomized rat bones.

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Workshop 6.2

Hormonally active agents and plausible relationships to adverse effects on human health*

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Abstract: A hormonally active compound was first identified in the book *Silent Spring* by Rachel Carson in 1962, implicating the effect of pesticides such as DDT and the derivatives. Nearly four decades later, the book *Our Stolen Future* by Theo Colborn et al., and other pertinent publications have revisited and broadened the issue regarding a variety of possible chemicals and the area exposed. Translation and publication became available in Japan within the last four years. Since then, Japan joined the member countries involved in the global issue of endocrine disruptors, the "environmental hormone".

Although a significant number of chemicals possessing a hormone-like action have been recognized for many years, and the action of their biological plausibility related to the receptor-mediated effects strongly suggests possible human effects comparable to hormonal changes in wildlife, little is known about evidences or adversities in experimental animals and humans. The most essential key to resolving these dilemmas may be to understand the mechanism of actions (i.e., a possible low-dose issue). In other words, the mechanism at the low-dose effect may be resolved simultaneously by the mechanism of three major questions linked to the low-dose issue; namely, threshold, possible oscillation, and additive and/or synergistic action.

INTRODUCTION

The objective of this paper is to summarize all currently available information on hormonally active agents and plausible relationships to adverse effects on human health from the standpoints of the mechanisms of action of these chemicals.

It is not uncommon to come across agrochemicals and industrial chemicals that have hormone-mimic effects. These chemicals, the so-called "environmental hormones", often accumulate at detectable levels in the environment, and it has been feared that they may have adverse effects not only on wildlife but also on human beings. Following reports of feminization and decreased colony size of wild creatures, and reports suggesting a possible association of these chemicals with abnormalities of reproductive organs and oncogenesis in humans, attention has focused on the possibility that these occurrences may be associated with exposure to endocrine-disrupting chemicals (EDCs). In this connection, we would like to draw the attention of the reader to a Japanese translation of the book *Our Stolen Future*, written by Theo Colborn et al.

*Report from a SCOPE/IUPAC project: Implication of Endocrine Active Substances for Human and Wildlife (J. Miyamoto and J. Burger, editors). Other reports are published in this issue, *Pure Appl. Chem.* 75, 1617–2615 (2003).

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This paper will review the subjects related to EDCs, the courses of arguments regarding the possible hazards of these chemicals, and current medical subjects pertaining to them.

CHEMICALS WITH HORMONE-MIMIC ACTIONS

Substances with hormone-mimic effects can be divided into four groups:

- hormones found in vivo;
- medicines with hormone-mimic actions manufactured for use in hormonal therapy, etc.;
- plant hormones known to exert phytoestrogen-like actions; and
- chemicals found in environments that can interact with hormone receptors.

In addition, substances that do not interact with hormone receptors but exert effects on gonads by their modifying effects on steroid metabolism may be deemed as hormone-mimics in the broader sense of the term. In this paper, however, emphasis shall be placed on the hormone-mimic actions mediated by receptors that play essential roles in the mechanism of actions of hormone-mimics.

CHARACTERISTICS OF THE RECEPTOR-MEDIATED ACTIONS OF HORMONE-MIMICS

The receptor-mediated actions of hormone-mimics are fundamentally characterized by the similarity in structures of the receptors involved, crossing the barrier of animal species. These characteristics allow us to speculate the possibility that the actions of these chemicals exerted in nature may also occur in humans.

Second, since similarities in the structure of various sex steroids and hormones are also known, it is possible that each individual hormone-mimic exerts diverse effects by acting on male hormone receptors, female hormone receptors, and nuclear receptors (including many orphan receptors), etc.

Third, many of these chemicals are excreted from the living body in the form of conjugated inactive substances instead of as degraded metabolites. They may also be eliminated in the unchanged form. Therefore, if feces and urine containing these substances are eliminated into river water, it is plausible to imagine that even inactivated hormones can sometimes become active and exert hormone-mimic actions in the environment. This is one of the characteristics unique to this class of chemicals.

Receptor-mediated responses involve many unresolved questions. Various undefined elements may be involved, including the relationship between receptor binding and signals, the relationship between receptor-ligand binding (ligand: substances that can bind to receptors) and the dissociation of ligands from receptors, signal cross-talks, involvement of unknown nuclear receptors, etc.

The actions of these chemicals add to the effects of intrinsic hormones. For this reason, these chemicals may exert their actions in a way different from that known for other chemicals that do not have structural or functional counterparts in vivo. For example, stimulation of hormone receptors by these extrinsic chemicals may modify homeostasis in vivo, leading to down-modulation of the physiological stimulation of these receptors by the intrinsic ligands. Therefore, the influence of the continued effects of environmental hormones needs special study.

PITFALL IN THE EFFECTS OF HORMONE-MIMICS

We must distinguish the interactions of endocrine hormone-mimics with hormone receptors from the hazards caused to endocrine tissue. Bearing this in mind, let us now summarize the problems related to the effects of hormone-mimics.

Antagonistic effects maintaining homeostasis

The endocrine system is regulated by homeostatic mechanisms. It is not uncommon for the effects of small amounts of hormone-mimics to interfere slightly with these mechanisms, often with no adverse influence; this is well known. However, this is not always the case. There seems to be a group of genes that act antagonistically to each other in the maintenance of homeostasis.

With the uterotrophic assay, which is used to check for estrogenic activity, the ovary is removed in advance and the blood level of the intrinsic female hormone is reduced to the minimum. Under the thus-created extremely shrinking state of the uterus, the test substance (a chemical or hormone) is administered to evaluate for its effects on the inflation of the uterus. This test (checking for growth of the uterus in ovariectomized animals) is designed to evaluate the hormone activity and effects of hormone-mimics under conditions of blockade of homeostasis.

This test method itself is valid. However, there is no sufficient rational evidence that indicates that the responses observed under such indirect control conditions of the living body can serve as an indicator of the health hazards of hormone-mimics. Although the ovo-testes seen in lower vertebrates may be used if the effects observed were to be valid as such an indicator, there is no consensus on what is valid as an indicator of the health hazards of EDCs when mammals are used as experimental animals.

Down-regulation of the expression of receptors

It is known that the expression of gene-encoding receptors is down-regulated by continuous stimuli, leading to reduced receptor activity. This can lead to a paradoxical outcome wherein the effects observed in the presence of low levels of a substance are not seen at high levels of the same substance. If this phenomenon occurred in individual organisms, the dose–response relationship will be nonlinear.

This means that extrapolation of results obtained at high levels of the chemicals, to conditions where low levels of the same substance are present, would be difficult. It is needed to test the validity of this hypothesis; analysis of the mechanisms underlying this phenomenon if the hypothesis were indeed valid, are thus important. Studies to resolve these questions are now under way.

Data gap concerning the effects of female hormones

In mature women, there are high levels of physiological hormones in vivo, and these are subject to cyclic control. It has been proposed that girls with inadequate physical growth begin menstruation at lower ages and undergo sexual maturation earlier than usual, and that hormone-mimics in these subjects can precipitate breast cancer.

The weak links in this hypothesis have been pointed out, and it has been shown experimentally that estrogen by itself may be teratogenic, although this tendency has been shown to be weak. It is known that organisms are programmed such that excessive exposure to estrogens during the intrauterine period or other developmental stages is avoided.

There are many open questions as to the mechanism by which mature females remain physiologically stable, even when exposed daily to high levels of estrogen (400 pM/l). Some additional dramatic effects may be needed to disturb this homeostatic physiology.

Multigeneration tests and effects on fetuses

It has been shown that exposure to hormones or hormone-mimics during intrauterine or early neonatal periods can lead to irreversible changes in the pattern of development. This susceptibility period is short, extending from the 13th gestational day to about one week after birth. These effects are the so-called "intrauterine window effects."

In animal studies involving observation of experimental animals for two or more generations, no effects of EDCs have been demonstrated. The question therefore arises as to why window effects are observed during the short period mentioned above. It is unknown whether or not these effects really do occur, and if they do, how they are produced.

Delayed growth of the thalamic nucleus specific to males (called sexual dimorphic nucleus) is seen in male rats treated with female hormones. We may say that under conditions of homeostasis of the physiological hormones in mature individuals, exposure to dose levels that usually cause only reversible changes can lead to irreversible changes, if the exposure occurs during genesis, morphogenesis, or functional development. However, there are no ample data endorsing this view in humans.

Considering the biological plausibility inferred from the experimental data accumulated to date*, we may say that there are no sufficient data that clearly rule out this view. Close attention has therefore been paid to these effects in children.

New theories of methodology, focusing on effects in fetuses and children, are now being developed, primarily in the United States, or the World Health Organization, within the framework of children's program, etc.

HEALTH HAZARDS AT LOW LEVELS OF EXPOSURE

Chemicals used for agriculture or industrial purposes are marketed, in general, only after their effects on living beings have been investigated. We may therefore understand that they are used on the premise that the possibility of these chemicals exerting hazardous effects on health at relatively high-dose levels has been almost ruled out. Nevertheless, problems with EDCs have begun to be highlighted. These problems may not be confined to those related to the accumulation of these substances through food chains in the ecosystem, but also to the additional possibility that these chemicals may exert effects at low-dose levels even if they have been declared safe at high-dose levels. The latter possibility may apply, however, only to some cases and not to others.

We may say that a major issue pertaining to EDCs that must be resolved urgently is whether or not they pose health hazards at low-dose levels. This issue can be summarized into the following three questions:

- presence/absence of threshold level;
- presence/absence of synergistic or additive effects; and
- possibility of extrapolation of high-dose effects to low-dose levels (i.e., presence/absence of a linear dose–response relationship).

No clear-cut answers have as yet emerged to these questions. Considering the above-mentioned characteristics of the effects of hormones, it is plausible to imagine how difficult it may be to resolve these questions.

To determine if these chemicals exerted hazardous effects on health at low-dose levels, the following basic questions may need to be considered; their biological plausibility is hardly denied.

- Regarding the presence or absence of threshold levels, it seems likely that many chemicals suspected of being EDCs can easily permeate across the cell membrane, which is composed of phospholipids. Therefore, assuming that one receptor molecule reacts with one chemical molecule, the lower limit of the dose level exerting the chemical's effects would be extremely low.

  Of course, since the probability of the binding of a ligand to the receptor will be low if the dose level is low, we cannot say that there is no threshold level for the effects seen in the low-

*Biological plausibility: Likelihood of a phenomenon as judged by considering the difference or similarity of elements of reactions in individual organisms, on the basis of the results of a series of related biological experiments. (Cf. probability)

dose-level range. In fact, for bisphenol A (which has been attracting close attention because of its hazardous effects on health at low-dose levels), the presence/absence of a threshold level has not yet been reported. It seems rational, therefore, to assume that these health hazards occur in a very low-dose-level range.

- If we consider not only the affinity of each substance for the receptor, but also the nonlinearity of responses (e.g., waveform responses as a result of reduced receptor expression following an increase in dose level), it is possible to assume that there are U-shaped or reverse U-shaped reactions, or oscillational dose–response curves. Interim data endorsing such a view are being accumulated.
- Regarding the possibility of synergistic or additive effects, the observation of additive effects among different nuclear receptors has been reported. Data yielded by analysis of interactions between receptor signals also suggest such a possibility. In fact, the dose–response curves for some composite materials were reported to be additive, but not synergistic.

Thus, the questions on health hazards at low-dose levels have several aspects:

- type of receptor-mediated actions of the hormone mimics;
- diverse reactive characteristics on the part of the receptors;
- diverse modification during expression of intracellular signals; and
- factors involved in irreversible changes related to morphogenesis and functional development.

Resolution of all these aspects of the question will lead to clarification of the mechanism of actions of the substances from each of the aforementioned standpoints. While these questions are among the hottest research themes at present, they are certainly unlikely to be resolved easily.

At a workshop held in North Carolina, USA, in October 2000, health hazards of chemicals at low-dose levels were discussed. Investigators for and against the possibility of these substances posing health hazards at low-dose levels gave detailed accounts of their studies, and no definitive conclusions could be reached, as the arguments of both sides appeared to be tenable.

This means that reports affirming the plausibility of these substances posing health hazards at low-dose levels in animal experiments cannot be immediately rejected. The workshop concluded by pointing out the necessity of paying attention to the possible hazards on fetuses and neonates.

HEALTH HAZARDS OF HORMONE-MIMICS TO HUMANS

The possibility of health hazards of hormone-mimics to human beings have not been supported by adequate epidemiological data, and the number of cases for which the data clearly endorse such effects is quite small. The U.S. National Research Council (NRC) emphasizes the necessity of conducting further epidemiological studies on this topic (NRC, 1999).

In conclusion, this paper summarizes the current knowledge concerning the health hazards of hormone-mimics to humans. Reports dealing with the effects of these substances on humans are confined to those pertaining to the effects of dioxins and polychlorinated biphenyls (PCBs); the validity and usefulness of these results have not yet been established.

The following information is based on case studies conducted to date.

Health hazards of dioxins

Regarding health hazards of dioxins, two-year dosing studies revealed weight loss and liver damage, and three-generation reproductive studies in rats disclosed intrauterine death and a decrease in litter size. Onset of endometriosis in rhesus monkeys has also been reported.

A causal relationship of EDCs to the following episodes in humans has been suggested: biased male-to-female ratio in children born in the dioxin-exposed Seveso area of Italy, and increased inci-

dence of cleft palate in the Diemerzeedijk district of the Netherlands, probably due to steroids. In both of these cases, the U.S. Environmental Protection Agency (USEPA) did not affirm a causal relationship, and classified them as cases requiring special attention.

No consensus has been reached concerning the relationship of hypothyroidism observed in the inhabitants along Lake Michigan to the ingestion of PBB- (polybrominated biphenyls-) contaminated fish.

Effects on mature females (e.g., increased incidence of breast cancer)

No reports affirm the effects of dioxins on mature human females (e.g., effects on breast cancer or endometriosis as discussed below). There are many unresolved questions on this topic. However, none of the studies conducted in mature experimental animals revealed data endorsing the plausibility of occurrence of such effects. On the other hand, it is known that the age at menarche is lower and the incidence of breast cancer higher in females exposed to dioxins. Some investigators cite these data when discussing the health hazards of dioxins. It is also known that females exposed to dioxins are often taller.

In European countries, a height increase of about 3.5 mm per year and an approximately one-year decrease in the age at menarche have been reported during the past 30 years. It is difficult to identify the influence of extrinsic endocrine factors on these changes, and no studies addressing this issue have been reported to date. Although several studies have been published concerning the effects of female hormone preparations, including pills used for contraception and hormone replacement therapy in postmenopausal women, no studies have provided data that establish the effects of EDCs.

Endometriosis

Endometriosis is a disease of unexplained origin that is seen in primates with sexual cycles. It has been pointed out that this disease tends to be more severe in individuals exposed to dioxins (2,3,7,8-tetrachlorodibenzo-*p*-dioxin [TCDD] and to PCBs). Data yielded from experiments in rhesus monkeys are used as evidence to corroborate the causal relationship between dioxins and endometriosis. Thus, we cannot rule out the biological plausibility of these effects. However, no reports affirming the causal relationship in humans have been published.

Possibility of other effects on humans

Biological plausibility has also been considered for the following effects of hormone-mimics on humans: qualitative dysfunction of human sperm, effects on neurobehavior of neonates, and immune functions. The effects on immune functions have been suggested by reports of cases with Yu-sho (PCB intoxication).

CONCLUSION

The International Program of Chemical Safety (IPCS), a section of the World Health Organization, has released a Web site publication "Global Assessment of the State-of-the Science of Endocrine Disruptors" (GAED), June 2002 (URL: <http://ehp.niehs.hih.gov/who/>). WHO/IPCS started the GAED program in March 1998 after the publication of *Our Stolen Future* (Theo Colbone et al., 1996). The publication took three years to edit; covering a policy to document all the published pertinent literatures, to summarize them as descriptive manner solely based on those published literatures. Twenty-seven expert scientists and 20 independent peer-reviewers participated in editing the GAED.

Other reports on nonylphenol and octylphenol, released by the Japanese Ministry of Environment (MoE), revealed an "ovotestes" formation that was observed in the assay of the laboratory experimen-

tal fish (*Medaka*) exposed to doses close to those recorded in the monitoring fields in the MoE surveillance. Further, phthalates, such as di-(2-ethylhexyl)phthalate, di-cyclohexylphthalate, and butylbenzylphthalate, as selected and prioritized chemicals by the MoE, showed some unique data in different endpoints, including mRNA expression, in dose ranges lower than those no observed effect levels (NOELs) and/or no observed adverse effect levels (NOAELs) reported previously.

The effects of EDCs on human health are unknown at this moment. However, due to the biologically plausible data currently accumulated, the existence of endocrine disruptions under certain circumstances seems to be a reality. Thus, by the time of the SCOPE/IUPAC symposium, the EDC research for the next stage may shift from plausibility to possibility, and put forward further mechanistic research.

Introduction:

Toxicogenomics
- a new paradigm of toxicology

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Summary. Molecular biology has enabled the elucidation of biological subjects with bilateral strategies, namely, an inductive approach and a deductive approach. Along with the development of the mouse whole-genome sequencing project, it has enabled elucidation of the science bilateral interrelationships between the toxicological phenotypes related to particular toxicants and expression profiles of pertinent genes induced by exposure to toxicants. While a conventional inductive approach permits exploration of the toxicological mechanism by cloning genes and analyzing gene and protein expression during the course of chemical exposure, the newly developed deductive approach potentially permits the elucidation of the toxicological phenotype(s) through gene expression.
Microarray technology has dramatically changed the time course of drug discovery in new drug development. Potential therapeutics can be screened for thousands of endpoints indicative of efficacy and adverse toxicity at one time using the microarray technology. Simultaneously, the same technology can be used to explore unique genomic "expression fingerprints", which can be used to group the biological effects of chemical actions at a various doses, time intervals, or target tissues, in a variety of animal species, into profiles as the bases of gene expression. Accumulation of the expression profiles (here and elsewhere) of whole genomes for reference chemicals for a variety of treatment conditions permits the establishment of an informatics profile (here and elsewhere) for reverse toxicology, which is conversely supposed to predict the toxicological phenotypes solely by analyzing gene expression. This translational introductory oversees the future prospects of how microarrays can be used in applied toxicology.

Key words. Toxicogenomics, DNA microarray, reverse science, reverse genetics, reverse toxicogenomics

DNA microarrays

As an introductory keynote to "Toxicogenomics", a discussion on what toxicogenomics can offer to conventional toxicology is given here in this

paragraph. Toxicogenomics is based on DNA microarray and DNA chip technologies that are similar to those in other genome science fields (Lovett, 2000; Hamadeh et al., 2001; Storck et al., 2002) i.e., the DNA microarray fixed with cDNA by a DNA spotter, and hybridized with fluorescence-labeled cDNAs from tissue samples (Schena et al., 1995, 1996), and the DNA chip, on which a number of oligonucleotide probes are photolithographically synthesized, followed by hybridization of biotiny lated cDNAs from samples (Fodor et al., 1993). Originally, DNA microarray and DNA chip technologies have been used to analyze a large number of gene expressions, and thus, have been applied to such functional genomics fields as transcriptomics (Storck, et al., 2002) pharmacogenomics (Lloyed A, 2000), mutagenomics (Aardema and MacGregor, 2002), oncogenomics (Herrmann, et al., 2001), pathogenomics (Liefers, et al., 2001), and predictive diagnostic medicine based on clinical prognosis (Nakamura, 2001), and specifically, the latter DNA chip technology is a potentially powerful tool for identifying DNA sequences, thus, such inductive information has been applied widely in the research for single nucleotide polymorphism, SNP, in a variety of drug-metabolizing enzymes, etc., to establish an individualized "tailor-made pharmacology".

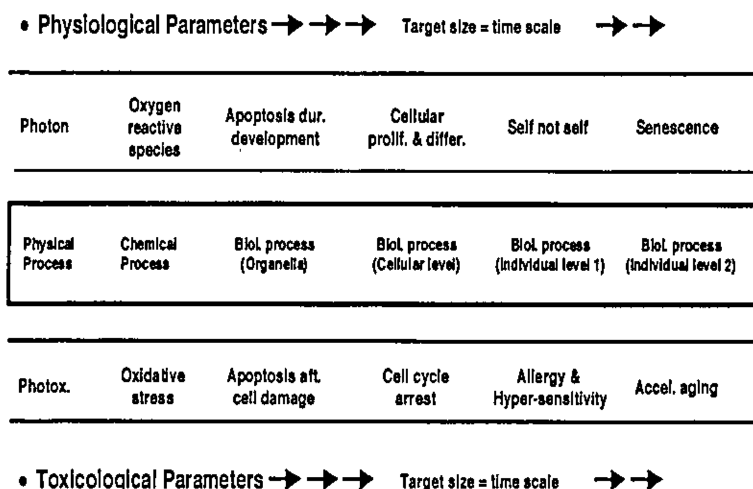

Fig. 1. Physiological parameters shown in the upper row vs. toxicological ones shown in the lower row along with the increase of participating target masses or time scales.

Microarray and /or DNA chip technologies applied in toxicology are called "toxicogenomics". Toxicogenomics can be applied bi-laterally, either inductively or deductively. Deductive approach of toxicogenomics shows a great, unexpected paradigm shift from conventional toxicology.

Toxicology and toxicogenomics

Before describing an over- view of toxicogenomics, what toxicology is, namely, its definition, entity, and scientific bases, should be reviewed first. Toxicology is an interdisciplinary area between biology/medicine and chemistry/physics. Key molecules participating in physiological responses and toxicological responses are presumably comparable (**Figure 1**), implying that physiological responses and toxicological responses may be a continuum. Pharmacology involves the identification of something available; on the other hand, toxicology involves the identification of not only the mechanism of toxicity but also clarifying a border of "nothing", i.e., NOEL, "no observed effect level", and/or NOAEL, "no observed adverse effect level". The goals of toxicology are to predict the effect of potential hazards on human health effects, and to identify the mechanism of toxicity, NOEL and/or NOAEL. In this regard, toxicogenomics is supposed to clarify comprehensively the border of "nothing". Although a prototype of "toxicogenomics" was developed in 1997 (Heller et al., 1997) to identify specific toxicological phenotypes, such as oxidative stress inducers, drug- metabolizing chemicals, and cell-cycle-specific modulators, comprehensive toxicogenomics became possible after the whole-genome sequencing project was accomplished in 2001. Because of the completion of the whole genome sequence, finally, the toxicology to predict "nothing" became possible.

Birth of reverse science & toxicology

In 1988, a new era of mouse genetics, reverse genetics, was started by generating the first knockout gene for mammalian species, murine int-2, by the group of Mario Capecchi's (Capecchi et al., 1988) and then Elizabeth Robertson's (Schwartzberg, et al, 1989). Thereafter, molecular biology has enabled the elucidation of biological subjects by bi-directional strategies, forward and reverse ones, i.e., the inductive and the deductive approach, respectively, where not only genes that possess a particular expression phenotype have been cloned by forward genetics, but also a number of genes of which functions were not known have been uncovered their function by reverse genetics, i.e., knockout technologies. The history of genetics teaches such bilaterally alternating strategies to strengthen scientific power. Thus, it is speculated that the inductive toxicology and deductive toxicology may complement each other.

Along with the development of the mouse whole-genome sequencing project, such bi-directional strategies for analysis became possible also in toxicology; the toxicologic phenotypes of particular toxicants and the expression profiles of pertinent genes reacting with the toxicants. While the inductive approach permits exploration of the toxicological mechanism by analyzing gene and protein expression during the course of toxicological testing, the deductive approach

permits prediction of the toxicological phenotype(s) solely by analyzing the gene expression. Microarray and/or DNA chip technologies have enabled the survey of a large number of gene expressions after exposure to a toxicant. Both inductive and deductive approaches have enabled application of DNA chip and/or the microarray in toxicological analysis, i.e., "toxicogenomics". Toxicogenomics enables exploration of the toxicological mechanism by analyzing a large number of gene chips inductively, and opens a new era of reverse toxicology, which is supposed to predict possible toxicologic phenotypes by distinguishing the expression patterns of particular genes from accumulated expression profiles. The DNA chip and the microarray technologies for the identification of specific toxicity groups are commercially available already, e.g., metabolic enzyme inducers, growth factor & receptor-mediated transducers, xenobiotic ligands for nuclear receptors, stress-response-gene modifiers, and cell-cycle regulator modifiers.

Reverse toxicology

Similar to reverse genetics, reverse toxicology is supposed to identify toxicological phenotypes solely by examining their expression profiles. Such deductive use of microarray technology for toxicology is called "Reverse Toxicogenomics", where it is expected to predict toxicological phenotypes solely by analyzing whole gene expression (**Figure 2**). This technique is requires a

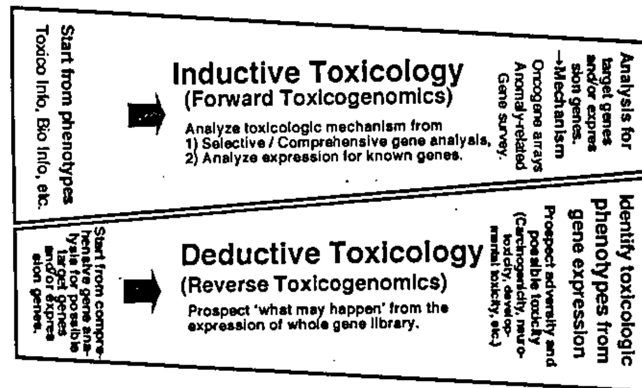

Fig 2. Structures of inductive toxicology vs. deductive toxicology. Former starts its analyses from toxicologic phenotypes toward the mechanism, whereas, the latter focuses in identigying toxicologic phenotypes solely from the gene expression profiling.