minimum number of animals, or even samples of in vitro-cultured cells after a relatively short period of exposure to a potential hazardous testing materials. The predictability by reverse toxicology depends upon the number of gene expression profiles accumulated, the number of phenotypes differentially linked to the gene expression profiles, and informatics linking such gene expressions and the phenotypes.

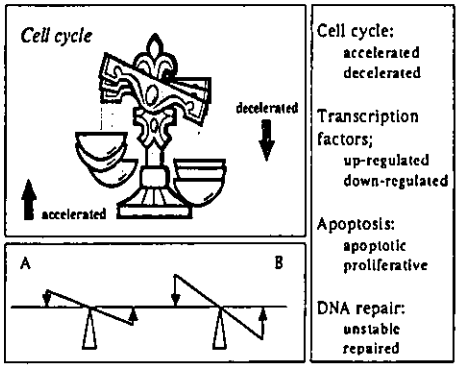
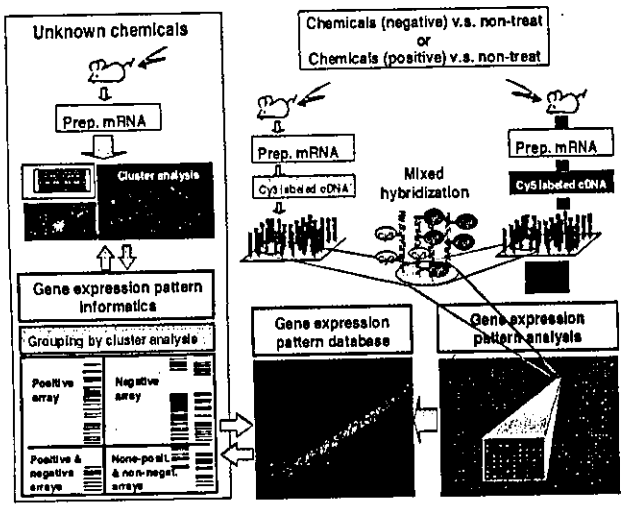


Fig. 3. Counter balancing gene expressions behind the homeostasis Visualization of different oscillatory balances A and B (left bottom).

At this moment, reverse toxicogenomics is still a theory. However, a variety of testing methods in toxicology will be replaced by reverse toxicogenomics, eventually. This strategy has the following advantages: it reduces the number of test animals, and the test period, and adopts simpler techniques by using established expression profile as new biomarkers rather than sophisticated methodologies requiring skill and experience. Furthermore, a specific proteome chip, expressing a series of specific genes, is supposed to function as "reverse proteomics" through a series of processes such as sample preparation, 2D gel electrophoresis, and mass-spectrometry for image analysis (Zhu et al., 2001). To set up an endpoint where NOEL or NOAEL exists, a traditional toxicology has been applied to incorporate something " invisible borders". Invisible borders are, in conventional toxicology, based on at least two major limitations: one in an endogenous factor(s) and the other in an exogenous factor. The former, for example, is hidden behind homeostasis, and the latter, for example, is behinbd a technological limitation. As shown in **Figure 3**, most living animals exhibit homeostasis between two (or more) counter- balancing vectors such as oxidation & reduction, apoptosis & anti-apoptosis, and acceleration & deceleration of cell cycle regulation. Since the counter-balancing counter-directional homeostasis, it appears static and one may not recognize the differences between one homeostatic stage, balanced at a low energy stage, to the other stage, balanced at a high energy stage (A and B in Figure 3). It is far more important to note that stage B is generally more risky. Toxicogenomics is expected to disclose such hidden homeostatic balances which are undetectable by conventional testing systems. The latter, an exogenous factor in a technological limitation, may be based on such resolution limit of light-microscopes, spectrophotometers, etc., and all



technologies exhibit their resolution point, i.e., "invisible barrier". Toxicogenomics also has a limit in terms of technical sensitivity; however, it may overcome presently available resolution limits in many ways, and hopefully identify a possible specific toxicological profiling.

Fig. 4. Practical toxicity-predicting system based on the gene expression microarray.

Practical approach

A sample of a chemical toxicity predicting system is shown in **Figure 4.** Mice are treated with, or without, a known toxic reference chemical (TRC), or treated with, or without, a known non-toxic reference chemical (NTRC). Then, the

Table 1: Possible toxicologic endpoints tested by in vitro or in vivo resources

Possibility of in vitro test	Toxicity Endpoint(s)
No [yes]*	*Morphogenesis, developmental anomaly*
No	*Identifying tissue-specific toxicity*
No/yes	*Epigenetic carcinogenesis (as modification of gene expression)*
	Metabolic activation
No	Hepatic activation leading to multi-tissue damage
Yes	Tissue-specific activation
	Receptor-mediated events
Yes/no	Neuronal tissue
Yes/no	Steroid hormonal tissue
Yes/no	Ah-receptors
Yes	*Cytotoxicity*
	Membrane activities
Yes	ion channels, ion pumps
	Inhibition of biochemical process
Yes	Uncoupling oxidative phosphorylation, inhibition of ATP production by redox cycling initiation to produce ROS
Yes	Oxidative phosphorylation to inhibit ATP production
Yes/no	Alteration of calcium homeostasis

*[yes]: Limited possibility at this moment, e.g. whole embryo culture.

messenger RNAs are extracted, and visualized with red color marker, cy3, for overexpression or with green color marker, cy5, for down-modulation. These color-labeled mRNAs will be processed into a competitive mixed-hybridization in a high-density hybridization array. Expression patterns are informatized in many ways. Along with accumulation of data to establish informatic profiles, specific gene clusters for TRC, NTRC, and those positive for both TRC + NTRC, andnegative for both TRC + NTRC, can be established. These databases can be compared with an expression profile that will be obtained from unknown chemicals (left box in Figure 4).

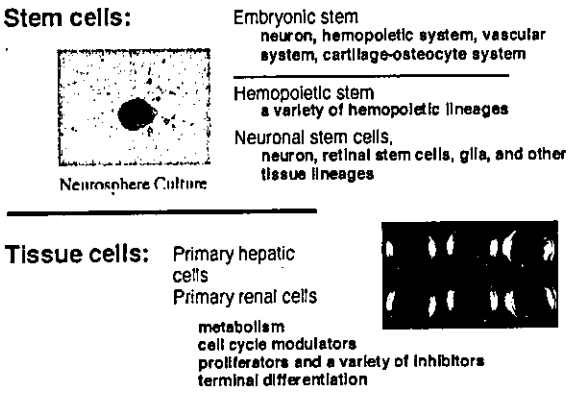


Fig. 5. A variety of in vitro resources for toxicologic gene expression array. Different cellular function for microarray analyses between stem cells and tissue cells. See text.

The technique requires only a limited number of animals, or even with cultured cells, in vitro, after relatively a short period of exposure. Depending upon the endpoints of toxicity aimed to focus on, even materials from in vitro culture may work efficiently (**Table 1**). As shown in Table 1, activities of membrane such as ion channels and ion pumps, the inhibitory effect of uncoupling oxidative phosphorylation, and the inhibition of ATP turnover by redox cycling, may be identified by the microarray. Possible toxicity related to developmental anomaly and morphogenesis (top of Table 1) may not be predictable by the use of in vitro cell culture; however, as seen in **Figure 5**, an in vitro system, for example, an embryonic stem (ES) cell, may predict some possible adverse effects of toxicity on the morphogenesis. Consequently, ES cells as well as hemopoietic and neuronal stem cells are particularly powerful tools for identifying the effect of toxicity on not only proliferation but also differentiation. Actually, one ES cell potentially corresponds to one individual; therefore, observing a microarray of ES cells may correspond to observing several millions of mice at the early developmental stage. Hepatic, renal and other types of primary cultured cells are limited but useful for observing such a variety of metabolic modulators, cell cycle regulators, and cell proliferation inhibitors and/or stimulators, in primary hepatic cells.

New paradigm of toxicology

Toxicogenomics, specifically reverse toxicogenomics, is about to open a new paradigm of toxicology from, at least, five aspects: first, the merging of such scientific borders as physiology and toxicology (also pharmacology and toxicology); second, a paradigm shift from "analog science" to "digital science"; third, visualization of hitherto unknown oscillatory changes behind homeostatic balances; fourth, comprehensive inter-species extrapolation; and lastly, a paradigm shift from inductive toxicology to deductive toxicology. As discussed previously in the first paragraph of this chapter, when we compare the aims of physiology, and toxicology, we find that they face opposite directions; however, participating molecules, seen in Figure 1, are presumably shared each other, physiologically as well as toxicologically, thereby implying that physiologic responses and toxicologic responses may be a continuum. In contrast, gene expression may not be continuum along with the dose response relationship, although a simple linear dose-response curve is generally accepted in the traditional toxicology. It appears to be clear that a different dose gives a different gene expression profile, in other words, the expression is expected to show not an analog change but a digital one, and a different dose behaves as a different chemical in the microarray. Although simple linear dose-response curves seem to apply in many cases, toxicological parameters may change discontinuously based on the genomic expression. This may be an advantage on one hand, because an appropriate array profiling of toxicologic responses can be eventually identified. On the other hand, there may still be a long way to go before reaching the final goal of defining the specific toxicologic array profiling for appropriate toxicologic phenotypes. The concept of safety borders, such as NOEL and NOAEL, may likely be re-established likely by means of such specific safety profiles. Visualization of invisible homeostatic balances is shown in the Figure 3. As was mentioned in the paragraph on oscillation strength, an additional new concept of "risk" may be re-established. Comprehensive 'interspecies'-extrapolation may be improved by informatics over different species such as mouse, rat, frog (Xenopus), and yeast. Interspecies extrapolation will be dealt with on a theoretical basis using a relatively small number of genes that are known to confer important allelic variations. This would result in better interspecies extrapolation, higher confidence of animal models, reduction in the number of animals needed for testing, shorter testing period, and most importantly, insights into pathways of toxicity and their mechanisms (US-EPA). Reverse toxicogenomics may be supported by the other four paradigm shifts mentioned above, and be used to predict toxicity and establish new concepts in risk assessment methodologies.

By means of toxicogenomics, we will be able to see a new toxicological world behind homeostasis and/or gene expression balance.

Acknowledgement: This review work was, in part, the product from the collaboration study with Yoko Hirabayashi, MD, PhD, Cellular & Molecular Toxicology Division, Center for Biological Safety and Research, NIHS, Japan.

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0013-7227/02/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 87(11):5325–5331

doi: 10.1210/jc.2002-012096

Progesterone Production and Actions in the Human Central Nervous System and Neurogenic Tumors

TSUKASA INOUE, JUN-ICHI AKAHIRA, TAKASHI SUZUKI, ANDREW D. DARNEL, CHIKA KANEKO, KAZUHIRO TAKAHASHI, MASAHITO HATORI, REIZO SHIRANE, TOSHIHIRO KUMABE, YOSHIMOCHI KUROKAWA, SUSUMU SATOMI, AND HIRONOBU SASANO

Department of Pathology (T.I., J.A., J.T., T.S., A.D.D., C.K., H.S.), Second Department of Surgery (T.I., Y.K., S.S.), and Departments of Applied Physiology and Molecular Biology (K.T.), Orthopedic Surgery (M.H.), and Neurosurgery (R.S., T.K.), Tohoku University School of Medicine, Sendai 980-8575, Japan

Progesterone has been suggested to be involved in the functions of the nervous system, but it has yet to be examined in humans. Progesterone has also been postulated to be involved in the biological behavior of various human neurogenic tumors via progesterone receptors A and B (PR-A and PR-B). In this study we examined the expression of PR and the enzymes responsible for progesterone biosynthesis (P450scc, 3β-hydroxysteroid dehydrogenase, and steroidogenic acute regulatory protein) in human brain. We also examined the distribution of PR isoforms in neurogenic tumors using immunohistochemistry and RT-PCR analysis. The presence of PR and mRNA for P450scc, 3β-hydroxysteroid dehydrogenase, and steroidogenic acute regulatory protein was detected in human brain. PR isoforms were detected in neurogenic tumors. PR-A and PR-B were equally expressed in meningiomas, but PR-B was the predominant isoform compared with PR-A in astrocytic tumors and Schwannomas. There was a statistically significant inverse correlation between PR-A and the proliferation index in meningiomas and astrocytic tumors. These findings suggest that progesterone is locally synthesized and exerts its actions through PR in the human central nervous system, and that progesterone may be involved in regulation of the growth and development of neurogenic tumors via PR, especially in the inhibition of tumor cell proliferation via PR-A. (*J Clin Endocrinol Metab* 87: 5325–5331, 2002)

PROGESTERONE HAS BEEN demonstrated to exert its effects not only in reproductive organs, such as the endometrium and breast, but also in several nonreproductive organs (1). Especially, progesterone has been reported to be synthesized in the central and peripheral nervous system and is considered to be a neurosteroid (2). The enzymes responsible for progesterone biosynthesis [cytochrome P450scc, which converts cholesterol to pregnenolone, and 3β-hydroxysteroid dehydrogenase (3β-HSD), which converts pregnenolone to progesterone] have been identified in the brain and spinal cord of the rat (2). These enzymes have been localized to the glial and Schwann cells of the rat nervous system (3, 4). In addition, the biosynthesis of steroid hormones, such as progesterone, is known to take place within the mitochondria. Steroidogenic acute regulatory protein (StAR) plays an important role in the process of steroidogenesis and appears to be the factor that results in the movement of cholesterol within the mitochondria (5), but its presence in the nervous system has been a topic of dispute. Progesterone synthesized in the nervous system is postulated to play essential roles in this system, including the regulation of myelin formation and the modulation of type A γ-aminobutyric acid receptor function (2, 6). Progesterone has also been found to activate myelin synthesis in the neurons of the rat (7). These relatively diverse effects of progesterone are all mediated through its binding to a specific intranuclear receptor, the progesterone receptor (PR) (8).

PR belongs to the nuclear receptor superfamily, and to date, two isoforms of this receptor have been identified, PR-A and PR-B (94 and 114 kDa in size, respectively) (9). The PR-B isoform is a full-length receptor, whereas the PR-A isoform lacks 164 amino acids in the N terminus of the PR-B isoform. Both PR-A and PR-B are derived from transcripts initiated from two distinct estrogen-inducible promoters within a single copy of the PR gene (10). Both isoforms have been demonstrated to function as ligand-activated transcription factors, but they are not always equal in their functional properties and progesterone actions (11). PR-B is, in general, transcriptionally more active than PR-A, but compared with PR-A, PR-B activity is also cell specific (12). In addition, the PR-A isoform has been demonstrated to repress the transcriptional activities of other steroid hormone receptors, including the estrogen receptor and PR-B (11, 13–15). Therefore, the relative levels of PR-A and PR-B within target cells may contribute to the nature and magnitude of functional responses to progesterone. Examination of the relative levels of these two isoforms in progesterone-responsive tissues, therefore, has become a very important topic of study, as it leads to a better understanding of progesterone actions.

In this study we first examined the presence of P450scc, 3β-HSD, StAR, and PR subtypes in the human central nervous system (CNS) to evaluate the status of possible progesterone biosynthesis and actions, *i.e.* whether progesterone may also serve as a neurosteroid in the human brain.

Progesterone has also been reported to influence the bio-

Abbreviations: CNS, Central nervous system; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; LI, labeling index; PR, progesterone receptor; StAR, steroidogenic acute regulatory protein.

logical behavior of human neurogenic tumors. For instance, the incidence of meningiomas is twice as high in females as in males (16), and their growth has been observed to be accelerated during pregnancy (17). To date, several investigations have examined the expression of PR in meningiomas and astrocytomas (18, 19). The presence of PR in meningioma has been reported to be involved in its histological features, tumor grade, and prognosis (18, 20, 21). In addition, an association between the histological grade for astrocytic tumors and PR has been reported (22, 23). These studies all suggest that progesterone may contribute to the proliferation of meningioma and astrocytic tumors via PR.

However, the expression of PR isoforms, PR-A and PR-B, has not been studied in neurogenic tumors. Therefore, in this study we examined the distribution of PR-A and PR-B in human neurogenic tumors using immunohistochemistry and RT-PCR analysis. We also examined the relationship between PR isoforms and the proliferation index (Ki-67), one of the most important clinicopathological parameters that describes the biological behavior of these tumors, to evaluate the possible biological significance of progesterone in the growth and development of these neurogenic tumors. In addition, we used RT-PCR analysis to evaluate the presence of cytochrome P450scc and 3β-HSD in these tumors to elucidate a possible mechanism of progesterone synthesis on tumor proliferation.

Materials and Methods

Tissues

Seventy-seven cases (36 males and 41 females) of neurogenic tumors were retrieved from the surgical pathology files at Tohoku University Hospital (Sendai, Japan). These tissues were obtained during surgery at Tohoku University Hospital between 1990 and 1999. The mean age of the patients was 48.6 ± 18.7 yr (range, 6–85 yr). All specimens for immunohistochemical examination were fixed in 10% neutral formalin for 18 h at room temperature and then embedded in paraffin. These specimens were subsequently sectioned at 3 μm and mounted onto silane-coated glass slides (Matsunami Co. Ltd., Tokyo, Japan).

Fresh-frozen specimens of both adult brain tissues (n = 5; 2 males and 3 females; mean age, 53.2 ± 15.6 yr) obtained from autopsy and neurogenic tumors (n = 27; 14 males and 13 females; mean age, 41.3 ± 19.0 yr) obtained from surgery were available for RNA extraction. Total RNA was extracted from tissues by the guanidine thiocyanate-cesium chloride method. Informed consent was obtained from the patients or the families of the patients. The ethics committee at Tohoku University School of Medicine (Sendai, Japan) approved this research protocol.

RT-PCR analysis

cDNA synthesis was performed using an RT-PCR kit (Superscript preamplification system, Life Technologies, Inc., Grand Island, NY). First-strand cDNA was synthesized from 1 μg total RNA in a 20-μl reaction volume containing 50 mM Tris-HCl (pH 8.3), 55 mM KCl, 3 mM $MgCl_2$, 0.02 M dithiothreitol, 0.5 mM deoxy-NTP, 62.5 mg/ml oligo(deoxythymidine), and 100 U Superscript II ribonuclease H^- reverse transcriptase at 42 C for 50 min. The reaction mixture was subsequently inactivated for 15 min at 70 C. An aliquot of each RT reaction product (1 μl) was amplified with gene-specific primers (Table 1) in a solution containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.1 mM deoxy-NTP, and 1.25 U *Taq* DNA polymerase (PCR Reagent System, Life Technologies, Inc.) in a total volume of 25 μl. PCR was performed on a DNA Engine Thermocycler (MJ Research, Inc., Cambridge, MA). A 35-cycle amplification profile consisted of denaturation at 94 C for 45 sec, annealing at 55 C for 30 sec, and extension at 72 C for 1.5 min. The amplified DNA products were then resolved on a 1.8–2.0% agarose gel and visualized by ethidium bromide staining. Negative controls without RNA and without reverse transcriptase were also included in the PCR reaction to test for exogenous DNA contamination. To assure that the PCR-amplified DNA observed in the agarose gel represented the gene-specific product of interest, we isolated, extracted, and purified the gene-specific bands of anticipated size from agarose gels, then cloned and sequenced them.

Primary antibodies

The primary antibodies used in the present study were hPRa7, hPRa2 (Neomarkers, CA), and Ki-67 (MIB-1, Immunotech, Marseilles, France). hPRa7 and hPRa2 are monoclonal antibodies raised in mice against PR isoforms obtained from a human endometrial carcinoma (EnCa 101). In a previous investigation we reported an immunohistochemical study using hPRa7 and hPRa2 (1). The hPRa7 antibody employed in this study recognized both PR-A and PR-B in immunoblot analysis (24). However, Mote *et al.* (25) reported that hPRa7 did not recognize PRB in their immunohistochemistry study of fixed tissues even after antigen retrieval, as evidenced by the absence of immunostaining for this antibody in the PR-B-expressing MDA-MB-231/PR-B cell line. This may be due to the inaccessibility of the epitope on PR-B recognized by hPRa7 in 10% formalin-fixed and paraffin-embedded tissue specimens (25), which, in turn, may be due to an alteration in the conformation of the molecule in such a way that the hPRa7 epitope is located in a manner that reduces its accessibility during immunohistochemistry. Ki-67 is monoclonal antibody that reacts with a nuclear protein expressed in the G_1, G_2, S, and M phases of the cell cycle (26). Therefore, immunostaining for Ki-67 is effective in predicting the biological behavior and prognosis of neoplasms of the nervous system (27, 28).

Immunohistochemistry

Immunohistochemistry was performed using the streptavidin-biotin amplification method employing a Histofine Kit (Nichirei, Tokyo, Japan), which has been previously described in detail (29). The hPR-7

TABLE 1. Oligonucleotide primer sequences used for RT-PCR analysis

mRNA	Primer	Sequence	GenBank/EMBL accession no.	Nucleotide no.
PR-AB	Sense	TGGAAGAAATGACTGCATCG	XM006190	1987–2182
	Antisense	TAGGGCTTGGCTTTCATTTG		
PR-B	Sense	ATGACTGAGCTGAAGGCAAAGGGT		176–605
	Antisense	CAAACAGGCACCAAGAGCTGCTGA		
P450scc	Sense	GCTGAGCAAAGACAAGAACA	M14565	1364–1545
	Antisense	GAATGAGGTTGAATGTGGTG		
3β-HSD	Sense	ATCCACACCGCCTGTATCAT	X53321	268–448
	Antisense	TCTGGATGATTTCCTTGTAGGAG		
StAR	Sense	GGCATCCTTAGCAACCAAGA	NM000349	388–586
	Antisense	TCTCCTTGACATTGGGGTTC		
GAPDH	Sense	TGAACGGGAAGCTCACTGG	M33197	731–1038
	Antisense	TCCACCACCCTGTTGCTGTA		

antibody was used at a dilution of 1:100, the hPR-2 antibody at 1:200, and the Ki-67 antibody at 1:50. The antigen-antibody complex was visualized with 3,3′-diaminobenzidine solution [1 mM 3,3′-diaminobenzidine, 50 mM Tris-HCl buffer (pH 7.6), and 0.006% H_2O_2] and counterstained with hematoxylin. Tissue sections of invasive ductal carcinoma of the breast were used as positive controls for PR isoforms. PBS (0.01 M) and normal mouse IgG were used instead of primary antibodies as a negative control. No specific immunoreactivity was detected in these tissue sections.

Scoring of immunoreactivity

All of the immunolabeled cells were evaluated as positive regardless of the degree of immunointensity (30). For statistical analyses of PR-A, PR-B, and Ki-67 immunoreactivity, we determined the percentage of positive cells in each tumor, as described by Sasano *et al.* (31) with some modification. Scoring of PR-A, PR-B, and Ki-67 in tumor cells was performed with high power fields (×400) using a standard light microscope. In each case more than 500 tumor cells were counted independently (double-blind) by two of the authors (T.I. and T.S.), and the percentage of immunoreactivity, *i.e.* the labeling index (LI), was determined. In our present study interobserver differences were less than 5%, and the mean of the two values was obtained.

Statistical analysis

LI values for PR-A, PR-B, and Ki-67 were presented as the mean ± 95% confidence interval. An association between PR and Ki-67 immunoreactivity was evaluated using a Spearman rank correlation. P less than 0.05 was considered significant.

Results

RT-PCR analysis

RT-PCR analysis revealed bands corresponding to the expected sizes for PR-AB (196 bp), P450scc (182 bp), 3β-HSD (181 bp), and StAR (199 bp) in human adult brain tissues, including cerebral cortex and cerebellum, regardless of age or gender. Breast carcinoma (PR-A and PR-B) and adrenal cortex (P450-scc, 3β-HSD, and StAR), employed as positive controls, were also shown to express the respective amplified gene product of the expected size. A band corresponding to the expected size for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 307 bp) was detected in all tissues examined in this study (Fig. 1).

RT-PCR analysis also revealed bands corresponding to the expected sizes for PR-AB and PR-B (429 bp) in human neurogenic tumors, as well as in breast carcinoma (Fig. 2), but

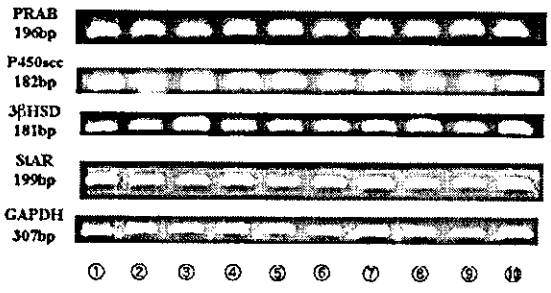

FIG. 1. RT-PCR analysis of human PR-AB, 3β-HSD, P450scc, and StAR. Bands corresponding to the amplified PCR products for PR-AB (196 bp) 3β-HSD (181 bp), P450scc (182 bp), and StAR (199 bp) were clearly detected in all human brain tissues we examined. GAPDH (307 bp), an internal positive control, was demonstrated in all tissues. 1–5, 70-yr-old female; 6–9, 66-yr-old male; 1, frontal lobe; 2, pons; 3, cerebellum; 4, temporal cortex; 5, hippocampus; 6, frontal lobe; 7, pons; 8, cerebellum; 9, temporal cortex; 10, positive control.

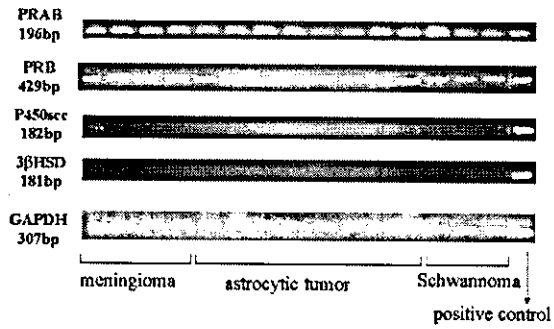

FIG. 2. RT-PCR analysis of human PR-AB, PR-B, 3β-HSD, and P450scc. Bands corresponding to the amplified PCR products for PR-AB (196 bp) and PR-B (429 bp) were clearly detected in all human neurogenic tumors we examined, but bands for 3β-HSD (181 bp) and P450scc (182 bp) were not detected in these tumors. GAPDH (307 bp), an internal positive control, was shown to be amplified in all tissues.

amplified gene products for P450scc and 3β-HSD were not detected. The sequences of all of these PCR products were confirmed by direct sequencing analysis (data not shown).

Immunohistochemistry

Immunoreactivity for PR isoforms was confined exclusively to the nuclei of tumor cells (Fig. 3). The median percentage of astrocytic tumor cells expressing PR-B was 88.4 ± 18.1%, whereas that expressing PR-A was 17.5 ± 21.0%. The median percentage of meningioma tumor cells expressing PR-B was 56.5 ± 27.3%, whereas that expressing PR-A was 41.6 ± 28.2%. The median percentage of Schwannoma tumor cells expressing PR-B was 46.6 ± 29.4%, whereas that expressing PR-A was 18.9 ± 18.8% (Table 2). The PR-A LI was higher in meningiomas than in astrocytic tumors and Schwannomas ($P < 0.05$). There was no significant correlation between PR isoform immunoreactivity, and gender or menopausal status in patients with human neurogenic tumors (Tables 3 and 4).

Immunoreactivity for Ki-67 (MIB1) was detected in the nuclei of tumor cells. There was a significant inverse correlation between PR-A and Ki-67 LIs in astrocytic tumors and meningiomas ($P < 0.05$; Fig. 4), but not between PR-B and Ki-67 LIs. No correlation was detected between either of the PR isoforms and Ki-67 LI in Schwannomas.

Discussion

A neurosteroid is defined as a steroid hormone produced from cholesterol in the nervous system, first proposed by Baulieu *et al.* (2). Progesterone has recently been reported to be one of the neurosteroids influencing various functions of both central and peripheral nervous systems as a modulator of neurotransmission (32). Progesterone is synthesized particularly in myelinating Schwann cells (3) and regulates myelin synthesis through PR in the nervous system by activating transcription (6). All studies to date, however, have previously been performed in the rat and mouse, and thus little is known about the status of progesterone biosynthesis, actions, and function as a neurosteroid in the human brain.

In our present study the number of cases examined was limited due to the nature of the study, but PR as well as the

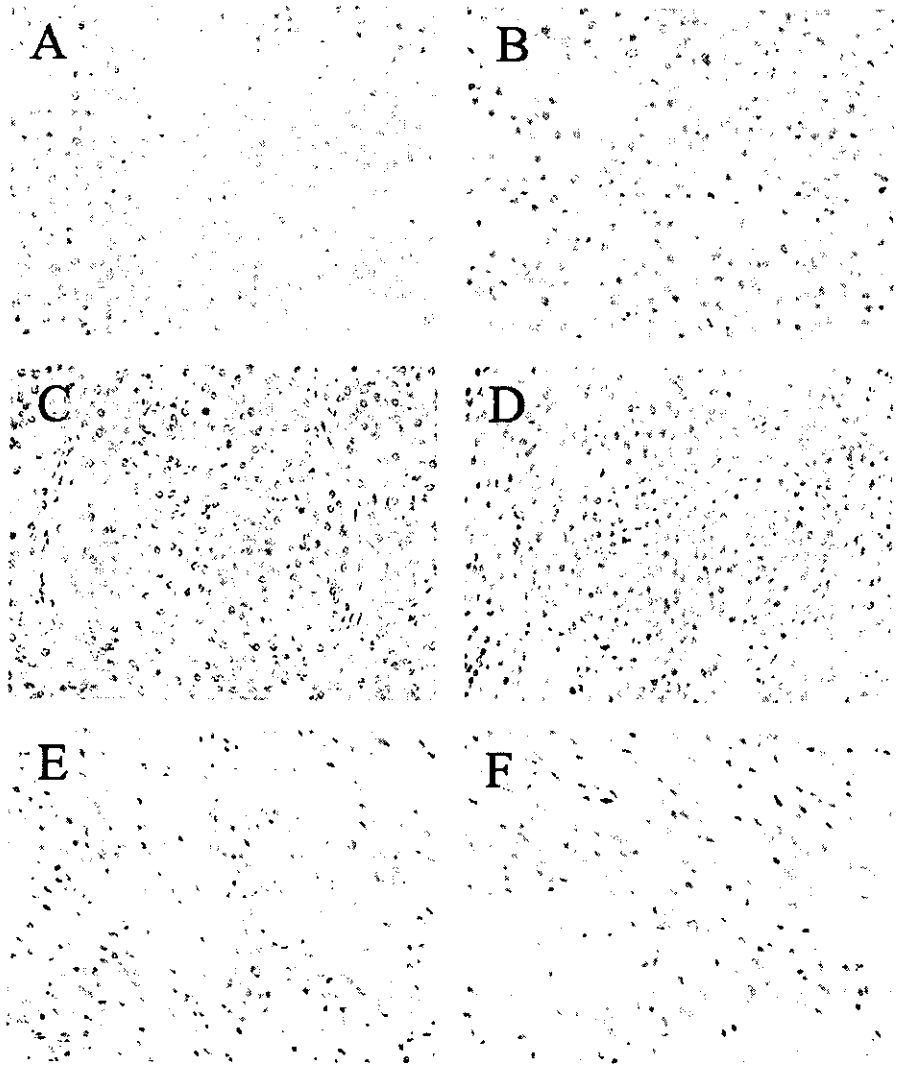

FIG. 3. Immunoreactivity for PR-A and PR-B in human neurogenic tumors. In astrocytoma, compared with PR-A (A), PR-B (B) was predominantly expressed. In meningioma, PR-A immunoreactivity (D) was as high as that of PR-B (E). In Schwannoma, compared with PR-A (G), PR-B (H) was predominantly expressed. A control study was performed using normal mouse IgG in astrocytoma (C), meningioma (F), and Schwannoma (I). Original magnification, ×400.

TABLE 2. The relative expression of PR-A and PR-B in the human neurogenic tumors

Neurogenic tumor	No.	Age (yr)	PR subtype	LI
Astrocytic tumor	26	38.2 ± 19.5	PR-A	17.5 ± 21.0%[a]
			PR-B	88.4 ± 18.1%
Meningioma	35	56.7 ± 15.7	PR-A	41.6 ± 28.2%[a,b]
			PR-B	56.5 ± 27.3%
Schwannoma	16	48.0 ± 16.0	PR-A	18.9 ± 18.8%[b]
			PR-B	46.6 ± 29.4%

[a,b] PR-A LI was higher in meningiomas than in astrocytic tumors and Schwannomas ($P < 0.05$).

enzymes involved in progesterone biosynthesis were detected in some regions of the human brain. Therefore, progesterone may also function in the human CNS as a neurosteroid. Robel and colleagues (32) reported that neurosteroids such as progesterone could influence memory processes by modulating type A γ-aminobutyric acid neurotransmission. Investigations by Baulieu *et al.* (6) and Chan *et al.* (33) reported that progesterone might enhance myelin formation of the nerve. Progesterone that is locally produced in the human brain may also promote new myelin sheath formation and contribute to regeneration and repair of the nerve cells, but further investigations are required to clarify the role that progesterone has as a neurosteroid in the human brain.

Two isoforms of human 3β-HSD, the enzyme responsible for converting pregnenolone to progesterone, have been identified to date, type I and type II. 3β-HSD type I is detected in the placenta and skin, whereas the type II isoform of 3β-HSD is detected in the adrenal glands, ovaries, and testes. Functionally, no differences are known to exist be-

TABLE 3. The percentage of PR isoforms in the human neurogenic tumors of male and female cases

Neurogenic tumor	PR subtype	Male	Female
Astrocytic tumor	PR-A	10.8 ± 10.7% (0–39.4%) (n = 15)	21.6 ± 24.4% (0–66.7%) (n = 11)
	PR-B	92.1 ± 7.2% (79.7–100%) (n = 15)	90.5 ± 8.8% (74.2–98.0%) (n = 11)
Meningioma	PR-A	40.3 ± 31.8% (0–81%) (n = 10)	42.1 ± 27.3% (0–77.5%) (n = 25)
	PR-B	65.1 ± 27.8% (0–91.7%) (n = 10)	53.1 ± 27.0% (0–90.1%) (n = 25)
Schwannoma	PR-A	22.0 ± 19.6% (0–46%) (n = 11)	12.1 ± 16.6% (0–32.6%) (n = 5)
	PR-B	56.5 ± 26.1% (0–91.4%) (n = 11)	24.6 ± 25.7% (0–57.7%) (n = 5)

TABLE 4. Correlation between PR isoform immunoreactivity and clinical parameters in the human neurogenic tumors

Neurogenic tumor	PR subtype	Prepuberial	Premenopausal	Postmenopausal
Astrocytic tumor	PR-A	33.9 ± 46.4%	21.1 ± 24.4%	14.4 ± 13.7%
	PR-B	80.2 ± 5.6%	95.2 ± 4.0%	88.2 ± 12.2%
Meningioma	PR-A	N/A	54.3 ± 11.0%	38.2 ± 29.9%
	PR-B	N/A	63.9 ± 8.2%	49.7 ± 30.0%
Schwannoma	PR-A	N/A	N/A	12.1 ± 16.6%
	PR-B	N/A	N/A	24.6 ± 25.7%

N/A, Not available for examination.

tween the two isoforms of 3β-HSD (34). In this study the sequences of the amplified PCR products for 3βHSD in human brain were consistent with 3β-HSD type II. Therefore, of the two isoforms of 3β-HSD, we believe it is the type II isoform of 3β-HSD that is expressed in the human nervous system. In this study StAR was also detected in the human brain. StAR plays an essential role in steroid hormone biosynthesis through the import of cholesterol into mitochondria (5). Previous studies have demonstrated the absence of StAR in human and rat brains (35, 36), but Furukawa and co-workers (37) have recently identified StAR in the rat brain. Results from our present study suggest that StAR may also be involved in the synthesis of progesterone in the human brain. Cholesterol is known to be transported into mitochondria via StAR. The conversion of cholesterol to pregnenolone via P450scc and that of pregnenolone to progesterone via 3β-HSD are likely to be one mechanism of progesterone synthesis in the human CNS. However, further investigations are required to fully characterize progesterone biosynthesis and actions in the human CNS.

Human neurogenic tumors have been demonstrated to express PR. However, the biological significance of PR in these tumors has not been fully characterized. The analysis of PR subtypes can provide new insights into the biological roles of various progesterone-responsive lesions, including neurogenic tumors. Immunohistochemical evaluation of receptor subtypes can provide important information in these studies. As described previously, Mote *et al.* (25) have demonstrated that the mouse monoclonal antibody hPRa7, which was also employed in this study, recognized only PR-A based on immunofluorescence immunostain analysis in MCF-7 M11/PR-A cells expressing only PR-A protein and PR-B-expressing MDA-MB-231/PR-B cells (25). They subsequently examined immunolocalization of PR-A and PR-B subtypes in human endometrium during the menstrual cycle using dual immunofluorescent histochemistry (25). However, it is also possible that MCF-7 M11/PR-A cells employed in the study by Mote *et al.* (25) contained protein or factors that blocked the PR-B-binding site of hPRa7. In addition, it is true that accessibility of the epitopes on antigen molecules may be influenced by various factors of tissue preparations, such as the duration of fixation and others. Therefore, further investigations are required to clarify the specificity of hPRa7 in recognizing PR-A in all of these 10% formalin-fixed and paraffin-embedded tissue sections, including those from neoplastic cases examined in this study. Our present study of immunohistochemistry of PR-A and PR-B subtypes was associated with limitation in interpreting the results as described above, but the PR-B LI was significantly higher than the PR-A LI in astrocytic tumors and Schwannomas, whereas the PR-B LI was as high as the PR-A LI in meningiomas. The discrepancy detected between these different types of neoplasms may represent a possible difference in the effects and/or roles of progesterone among different subtypes of human neurogenic tumors. Previous studies describing PR expression have demonstrated that the relative distributions of PR-A and PR-B in uterine leiomyoma, malignant endometrium, ovarian cancer, and breast cancer may be associated with the biological behavior and/or malignant grade of the tumor (38–42). Several investigators have reported that there was a significant correlation between histological grade and PR level in meningiomas and astrocytic tumors (18, 20–23). In meningiomas, aggressive biological behavior, including frequent recurrences and infiltrative growth associated with or without bone destruction, is highly correlated with negative PR status (20, 21), whereas high grade astrocytic tumors are correlated with the presence of PR (22).

Brain tumors are, in general, known to not metastasize outside of the cranial cavity, and thus, the status of cell proliferation is considered the most important predictor of biological behavior of these tumors. However, the correlation between the proliferation of tumor cells and PR isoforms

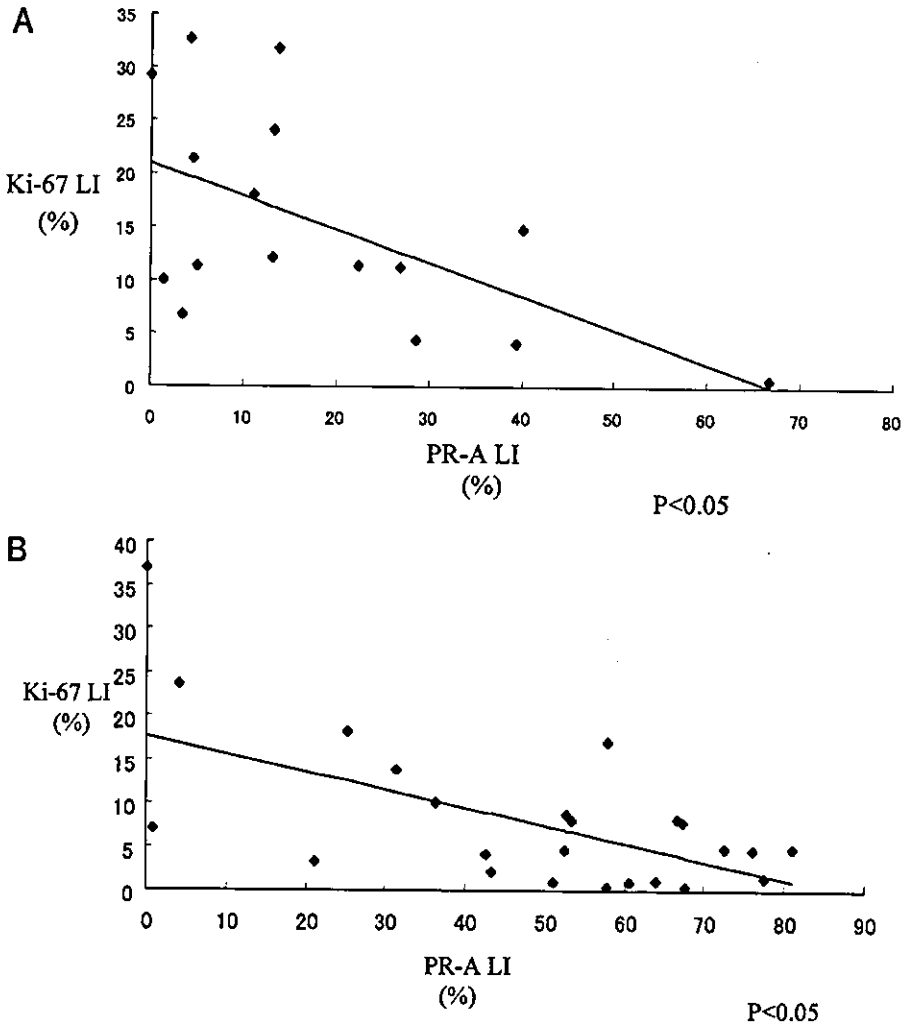

FIG. 4. Correlation between PR-A LI and Ki-67 LI in astrocytic tumors (A) and meningiomas (B). A, There was a significant inverse correlation between PR-A LI and Ki-67 LI in astrocytic tumors ($P < 0.05$). B, There was a significant inverse correlation between PR-A LI and Ki-67 LI in meningiomas ($P < 0.05$).

has not been examined in these tumors. In our present study a significant inverse correlation between PR-A level and proliferation index was detected in meningiomas and astrocytic tumors. Michelsen *et al.* (17) reported the growth of neurogenic tumors that were accelerated during pregnancy. Therefore, it is likely that a female sex steroid hormone may be playing an important role in the proliferation of neurogenic tumors, possibly acting as a growth factor. These data also suggest that progesterone contributes to the cell proliferation and development of neurogenic tumors via PR-A and PR-B. Therefore, progesterone may promote the growth of tumor cells via PR-B while inhibiting the growth via PR-A in meningiomas and astrocytic tumors. An introduction of selective progesterone receptor modulators can therefore contribute to the treatment of these tumors in the future. We could detect the presence of mRNA for 3β-HSD and P450scc, both of which are required for the synthesis of progesterone in the human nervous system. However, neither 3β-HSD nor P450scc immunoreactivity or protein was detected in these tumors in these tissues. Therefore, progesterone, in addition to its presence in the circulation, may be synthesized in the nonneoplastic nervous tissue surrounding these neurogenic tumors and may exert its effects through PR in neurogenic tumors.

In summary, progesterone may be synthesized and may act through PR in the human nervous system, possibly as a neurosteroid, and may also be involved in regulation of the growth and development of human neurogenic tumors via its binding to PR.

Acknowledgments

Received January 3, 2002. Accepted August 16, 2002.

Address all correspondence and requests for reprints to: Tsukasa Inoue, M.D., Department of Pathology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan. E-mail: tsukasai@gonryo.med.tohoku.ac.jp.

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Health Hazards of Endocrine-Disrupting Chemicals on Humans as Examined from the Standpoints of Their Mechanisms of Action

JMAJ 46(3): 97–102, 2003

Tohru INOUE*, Katsuhide IGARASHI and Jun SEKIZAWA

Chairperson, Biological Safety Research Center, National Institute of Health Sciences*

Abstract: Hormonally active compounds were first recognized in "*Silent Spring*" by Rachel Carson in 1962, which implicated pesticides, such as DDT and derivatives. Nearly four decades later, the book "*Our Stolen Figure*," by Theo Colborn *et al.*, and other pertinent publications have revisited and broadened the issue to a variety of chemicals and areas exposed. Translations of these books have just become available in Japan in the past three or four years, and since then Japan has started to join the debate and/or discussion of the global issue of endocrine disruptors—"Environmental Hormones." Although significant numbers of chemicals possessing a hormone-mimicking action have been recognized for many years and based on biological plausibility their receptor-mediating effects strongly suggest effects in humans similar to those seen in wildlife, little is known about the experimental evidence related to human adverse effects. The key issue in resolving the dilemmas posed by the biological plausibility and poor experimental evidence may be to clarify their mechanism of actions at low levels. In other words, the mechanisms of the possible low-dose effects may be resolved simultaneously by defining three major properties threshold, oscillation, and additive-synergism.

Key words: Receptor; Hormone mimics; Homeostasis; Effects at low dosage; Human hazards

Introduction

The objective of this paper is to summarize all the currently available information on the possible hazards of endocrine-disrupting chemicals (EDs) on human health from the stand-

This article is a revised English version of a paper originally published in the Journal of the Japan Medical Association (Vol. 127, No. 2, 2002, pages 197–201).
The Japanese text is a transcript of a lecture originally aired on October 12, 2001, by the Nihon Shortwave Broadcasting Co., Ltd., in its regular program "Special Course in Medicine".

points of the mechanisms of actions of these chemicals.

It is not uncommon to come across agrochemicals and industrial chemicals that have hormone-mimicking 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 on living 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 human, attention has been focused on the possibility that these occurrences may be associated with exposure to EDs. In this connection, a Japanese translation of the book entitled "*Our Stolen Future*," written by Theo Colborn *et al.*, was published some time ago.

This paper will review the problems related to EDs, the courses of arguments regarding the harmful effects of these chemicals, and current medical topics pertaining to them.

Chemicals with Hormone-Mimicking Actions

Substances with hormone-mimicking effects can be divided into four groups: (1) hormones found *in vivo*, (2) medicines with hormone-mimic actions manufactured for use in hormonal therapy, etc., (3) plant hormones known to exert phytoestrogen-like actions, and (4) chemicals found in environments that can interact with hormone receptors.

In addition, substances which 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-mimicking actions mediated by receptors which 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 the structures of the receptors involved, crossing the barrier of species. This characteristic allows us to estimate the possibility of the actions of these chemicals exerted in nature also occurring in humans.

Secondly, since similarities in the structure to 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, receptors in the nuclei (including some unknown receptors), etc.

Thirdly, many of these chemicals are eliminated 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 which 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 weakening of the physiological stimulation of these receptors by the intrinsic substances. 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.

1. Antagonistic effects on the maintenance of 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 uterus growth test, 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 undeveloped state of the uterus, the test substance (a chemical or hormone) is administered to check for its effects on the growth 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 ovotestes 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 ED's when mammals are used as experimental animals.

2. Down-regulation of the expression of receptors

It is known that the expression of genes encoding receptors is down-regulated by stimuli, leading to reduced receptor sensitivity. 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 non-linear.

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, and 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.

3. 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 dramatic effects are probably needed to disturb this physiology.

4. Multi-generation 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 EDs 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 are they produced.

Delayed growth of the thalamic nucleus specific to males (called sexual type II 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,[*1] 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 the effects in fetuses and children, are now being developed, primarily in the United States, 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 EDs have begun to be highlighted. These problems may be not confined to those related to the accumulation of these substances through food chains in the ecosystem, but also to the possibility additionally 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 EDs 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: (1) presence/absence of threshold level, (2) presence/absence of synergistic or additive effects, and (3) 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.

[*1] 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 a related biological experiments. (cf. probability)

(1) Regarding the presence or absence of threshold levels, it seems likely that many chemicals suspected of being EDs 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 very 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 dose level range. In fact, for bisfenol 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.

(2) If we consider not only the affinity of each substance for the receptor, but also the non-linearity 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.

(3) 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: (1) the type of receptor-mediated actions of the hormone mimics, (2) diverse reactive characteristics on the part of the receptors, (3) diverse modification during expression of intracellular signals, and (4) 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 on Humans

The possibility of health hazards of hormone-mimics on humans 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 US National Research Council emphasizes the necessity of conducting further epidemiological studies on this topic (National Research Council, 1999).

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

The following are based on case studies conducted to date.

1. 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 EDs 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 incidence of cleft palate in the Diemerzeedijk district of the Netherlands, probably due to steroids. In both of these cases, the Environmental Protection Agency (EPA) of the United States did not affirm a causal relationship, and treated 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.

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

No reports affirming 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 a number of 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 EDs.

3. 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 (TCDD/PCBs). Data yielded from experiments in rhesus monkeys are used as evidence to corroborate the causal relationship between dioxins and endometriosis. We cannot thus rule out the biological plausibility of these effects. However, no reports affirming the causal relationship in humans have been published.

4. Possibility of other effects on humans

Biological plausibility has been pointed out also on 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).

0013-7227/03/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 88(5):2309–2317

doi: 10.1210/jc.2002-021353

Sex Steroid Hormone Receptors in Human Thymoma

HIRONORI ISHIBASHI, TAKASHI SUZUKI, SATOSHI SUZUKI, TAKUYA MORIYA, CHIKA KANEKO, TOUICHIROU TAKIZAWA, MAKOTO SUNAMORI, MASASHI HANDA, TAKASHI KONDO, AND HIRONOBU SASANO

Department of Pathology, Tohoku University School of Medicine (H.I., T.S., T.M., C.K., H.S.); Department of Thoracic Surgery, Institute of Development, Aging, and Cancer, Tohoku University (S.S., T.K.); and Department of Surgery, Sendai Kousei Hospital (M.H.), Sendai 980-8575, Japan; and Departments of Pathology (T.T.) and Thoracic Cardiovascular Surgery (H.I., M.S.), Graduate School, Tokyo Medical and Dental University, Tokyo 113-0034, Japan

In this study we examined the immunohistochemical localization of sex steroid receptors for estrogen α (ERα) and ERβ, progesterone-A (PR-A) and PR-B, and androgen (AR) in human thymoma (n = 132) and correlated these findings with various clinicopathological parameters. We used RT-PCR and real-time PCR to further study the expression of these receptors in 20 thymoma cases. Immunoreactivity for all sex steroid receptors was detected in the nuclei of thymoma epithelial cells. The percentage of immunopositive cases and the H-score values for each receptor (mean ± SD) were: ERα, 66% and 85.8 ± 80.2; ERβ, 7% and 7.2 ± 8.7; PR-A, 4% and 2.7 ± 4.9; PR-B, 49% and 55.8 ± 68.3; and AR, 15% and 14.1 ± 11.7, respectively. The results of real-time PCR were consistent with those of immunohistochemistry, especially results for ERα, PR-B, and AR. A significant positive correlation was detected between immunoreactivity for ERα and PR-B. ERα immunoreactivity was inversely correlated with tumor size, clinical stage, WHO classification, and Ki-67 labeling index. In addition, the status of ERα immunoreactivity was significantly associated with a better clinical outcome in thymoma patients. Results from our study suggest that estrogens may inhibit thymoma growth via ERα, and that ERα immunoreactivity may act as a prognostic factor in human thymoma. (*J Clin Endocrinol Metab* 88: 2309–2317, 2003)

IT IS WELL known that sex steroids, such as estrogen, progesterone, and androgen, exert a wide variety of effects on target tissues in humans (1). The biological effects of sex steroid hormones are, in general, mediated through initial interactions with their native receptors. These receptors, including estrogen receptor α (ERα) (2), ERβ (3), progesterone receptor-A (PR-A), PR-B (4), and androgen receptor (AR), belong to a family of nuclear hormone receptors (1, 5). Steroid receptors are generally known to function as dimers and to activate transcription in a ligand-dependent manner by binding to the responsive elements located in the promoter region of various target genes (5). A better understanding of steroid actions in normal and neoplastic human tissues can be obtained by analyzing the expression of specific steroid receptors in these tissues.

In a study by Anser-Ahmed *et al.* (6), sex steroid hormones were shown to have important roles in regulating the function of the rat thymus. Estrogen has also been reported to be involved in the processes of development and atrophy of the mouse thymus (7–9). In addition, ER was detected in the homogenates of mature human thymus (10, 11), and recent investigations using ER knockout mice have indicated that the growth and maturation of the thymus were at least in part under the regulation of estrogen (7–9). Considering that sex steroids regulate cell proliferation not only in normal tissues but also in various neoplasms derived from hormone-dependent tissues, such as breast and prostate (12, 13), it is reasonable to postulate that sex steroids may be involved in the development of human thymomas. However, the status of sex steroid receptors has not been examined in human thymomas, and thus, the biological significance of sex steroid actions remains unknown in human thymoma tissues. Therefore, in the present study we studied the immunolocalization of ERα, ERβ, PR-A, PR-B, and AR in 132 cases of human thymoma tissues and correlated these findings with various clinicopathological parameters. Using RT-PCR and real-time PCR, the gene expression of these receptors was also analyzed in 20 cases of thymoma to further characterize these receptors in human thymoma.

Abbreviations: AR, Androgen receptor; ER, estrogen receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ir, immunoreactivity; LI, labeling index; PR, progesterone receptor.

Patients and Methods

Patients and tissue specimens

One hundred thirty-two cases of thymoma were retrieved from surgical pathology files at Sendai Kosei Hospital (Sendai, Japan) and Tokyo Medical and Dental University (Tokyo, Japan). All specimens were fixed for 24 h in 10% formalin and embedded in paraffin wax. The clinical data, including patient age, sex, menopausal status, the presence or absence of myasthenia gravis, tumor size, clinical stage (14), and WHO classification (15), are summarized in Table 1A.

Freshly frozen specimens were also available for RT-PCR analysis and real-time PCR in 20 cases (Table 1B). Specimens for RNA isolation were immediately frozen in liquid nitrogen and stored at −80 C until used for RNA isolation. RNA was extracted within 2 wk after surgery.

The research protocols used in this study were approved by the ethics committee at Tohoku University School of Medicine, Sendai Kosei Hospital, and Tokyo Medical and Dental University School of Medicine.

Antibodies

The antibodies used in this study are summarized in Table 2. Antibodies for the two isoforms of PR, PR-A (hPRa7) and PRB (hPRa2), were purchased from NeoMarkers Co. Ltd. (Fremont, CA). These monoclonal antibodies were raised in a mouse against PR isoforms obtained from a human endometrial carcinoma (EnCa 101). Clarke *et al.* (16) reported that the hPRa7 antibody recognizes both PR-A and PR-B in immunoblot

TABLE 1A. The summary of clinical data in 132 patients with thymoma examined in this study

Patients age (yr)[a]	54.0 ± 15.1
Sex	
Male	58 (43%)
Female	74 (57%)
Premenopausal	38 (29%)
Postmenopausal	36 (28%)
Myasthenia gravis	
(+)	24 (18%)
(−)	108 (82%)
Tumor size (mm)[a]	60.0 ± 25.5
Clinical stage	
I	67 (51%)
II	28 (21%)
III	21 (16%)
IV	16 (12%)
WHO classification	
A	28 (21%)
AB	23 (17%)
B1	29 (22%)
B2	39 (30%)
B3	13 (10%)

[a] Values represent mean ± SD. Other values represent the number of cases and percentages.

TABLE 1B. The summary of clinical data in 20 patients with thymoma for quantitative PCR

Patients age (yr)[a]	54.4 ± 13.2
Sex	
Male	10 (50%)
Female	10 (50%)
Premenopausal	38 (29%)
Postmenopausal	36 (28%)
Myasthenia gravis	
(+)	3 (15%)
(−)	17 (85%)
Tumor size (mm)[a]	61.3 ± 17.0
Clinical stage	
I	10 (50%)
II	4 (20%)
III	3 (15%)
IV	3 (15%)
WHO classification	
A	4 (20%)
AB	4 (20%)
B1	4 (20%)
B2	5 (25%)
B3	3 (15%)

[a] Values represent mean ± SD. Other values represent the number of cases and percentages.

analysis. However, using immunohistochemistry, Mote *et al.* (17) reported that hPRa7 did not recognize PR-B in fixed tissues even after antigen retrieval, as evidenced by the absence of immunostaining by this antibody in the PR-B-expressing MDA-MB-231/PR-B cell line. This is believed to be due to the inaccessibility of the epitope on PR-B recognized by hPRa7 in 10% formalin-fixed and paraffin-embedded tissue specimens (17).

Immunohistochemistry

Immunostaining was performed by using the streptavidin-biotin amplification method using a Histofine Kit (Nichirei Co. Ltd., Tokyo, Japan) for ERα, PR-A, PR-B, AR, and Ki-67, and EnVision$^+$ (DAKO Corp., Carpinteria, CA) for ERβ. Sections were heated in an autoclave at 120 C for 5 min in citric acid buffer (2 mM citric and 9 mM trisodium citrate dehydrate, pH 6.0) after deparaffinization for antigen retrieval. The slides were incubated with primary antibodies for 12–18 h in a moist chamber at 4 C. The dilutions of primary antibodies used in our study

TABLE 2. Characteristics of primary antibodies used in immunohistochemistry

Antibody	Source	Optimal dilution
ERα: NCLER-6F11 (monoclonal)	Novocastra Lab. (Newcastle, UK)	1:50
ERβ: 06-629 (polyclonal)	Upstate Biotechnology Co. Ltd. (New York, NY)	1:50
PR-A: hPRa7 (monoclonal)	NeoMarkers Co. Ltd. (Fremont, CA)	1:200
PR-B: hPRa2 (monoclonal)	NeoMarkers Co. Ltd. (Fremont, CA)	1:200
AR: AR441 (monoclonal)	DAKO Corp. (Carpinteria, CA)	1:100
Ki-67: MIB-1 (monoclonal)	Immunotech (Marseilles, France)	1:50

are summarized in Table 2. The antigen-antibody complex was subsequently visualized with 3,3′-diaminobenzidine solution [1 mM 3,3′-diaminobenzidine, 50 mM Tris-HCl buffer (pH 7.6), and 0.006% H_2O_2] and counterstained with hematoxylin. Tissue sections used as positive controls in this study were as follows: breast cancer for ERα, nonpathological breast for ERβ, endometrium for PR-A and PR-B, and prostate for AR. As a negative control for monoclonal antibody staining, normal mouse IgG was used instead of the primary antibodies. No specific immunoreactivity was detected in these sections. ERβ (Upstate Biotechnology, Inc., Lake Placid, NY) is a polyclonal antibody raised in rabbits. An 18-amino acid peptide from the N terminus of ERβ (amino acids 48–65) was made by solid phase synthesis on a multiple antigenic peptide backbone (Protein and Carbohydrate Structure Facilities, University of Michigan, Ann Arbor, MI). This antibody was characterized and reviewed for specificity using immunohistochemistry, including the preabsorption test and immunoblotting by Mitchner *et al.* (18). Because this antibody was unavailable, we used a normal rabbit IgG antibody instead of the primary antibody as a negative control for immunohistochemistry of ERβ. No specific immunoreactivity was detected in these tissue sections.

Scoring of immunoreactivity

For semiquantitative analysis of immunoreactivity of steroid receptors, H-score (19) was used in this study. Briefly, more than 500 tumor cells were counted in each case, and the H-score was subsequently generated by adding the percentage of strongly stained nuclei (3×), the percentage of moderately stained nuclei (2×), and the percentage of weakly stained nuclei (1×), giving a possible range of 0–300. The score was independently obtained by two of the authors (H.I. and T.S.) after determining the areas of immunostained slides using a double-headed light microscope. In the present study interobserver differences were less than 5%, and the mean of the two values was obtained. Cases that were found to have an H-score more than 50 were noted as steroid receptor-positive thymomas, according to a report by Thike *et al.* (20).

Ki-67 immunoreactivity was scored in more than 500 tumor cells for each case, and the percentage of immunoreactivity regardless of the immunointensity, *i.e.* labeling index (LI), was determined in the manner described for steroid receptor immunoreactivity. Ki-67 LI indicates proliferative activity in various neoplastic tissues, including thymoma (21).

RT-PCR and real-time PCR

RT-RCR and real-time PCR analysis for ERα, ERβ, PR-A, PR-B, or AR was performed in 20 cases of thymoma. Total RNA was extracted from frozen samples of thymoma with TRIzol reagent, according to the manufacturer's instructions (Life Technologies, Inc.-BRL, Grand Island, NY). RNA concentrations were determined spectrophotometrically, and all RNA samples were stored at −80 C until used for cDNA synthesis. An RT kit (SuperScript Preamplification system, Life Technologies, Inc.-BRL) was used in the synthesis and amplification of cDNA.

Total RNA (5 μg) was denatured at 70 C for 10 min and then reverse transcribed in the presence of 25 ng/μl oligo(deoxythymidine) primer (Life Technologies, Inc., Tokyo, Japan), 2.5 mM $MgCl_2$, 0.5 mM deoxy-