factors, diet, alcohol use, smoking, medical disorders, and concomitant use of medications. The findings of post-marketing observational studies are thus vital for determination of the appropriate use of drugs. In addition, effective and prompt communication of these findings is essential so that the information needed at the medical frontline is supplied to ensure that package inserts, containing basic pharmaceutical data, function as real-time sources of information. To this end, the PMDA framework should be reinforced and detailed post-marketing information gathered for analyses of the pooled data.

NON-CLINICAL SAFETY ASSESSMENT OF METABOLITES

Efforts in Japan

At present, no guidance is available in Japan on the performance of toxicity studies with drug metabolites, so these are handled on a case-by-case basis. The basic viewpoint is that safety verification is required in cases such as the following: (1) those in which metabolites not found in animals occur in humans, (2) those in which the ratios of the metabolites formed in humans and animals differ and metabolites are formed in greater numbers in humans (and where safety cannot be assured on the basis of the metabolite exposure in animal studies), and (3) those in which metabolites considered to have very strong pharmacological or toxicological effects are formed. In cases in which the metabolite of concern is a known substance and its safety profile is clear, or in which the metabolite concerned is deemed to be pharmacologically inactive, or in which the amount formed within the body or exposure to it is believed to be very low, verification of the safety of metabolites may not be required.

The data for new drugs approved in recent years (2000 ~ 2006) were investigated as to the safety assessment of metabolites, study details and rationales, and trends toward assessment of the safety of metabolites. It was found that metabolite safety testing had been conducted on 48 (39%) of the 123 drugs containing new active ingredients (excluding recombinant drugs and biological preparations etc.). Of the 48 products that were investigated for metabolite safety, 44 single-dosing studies (92%) were performed, 7 repeat-dosing studies (15%), and 15 genotoxicity studies (31%) (with some duplication). The most common reason for performing the studies which was given for 44 studies was "It is the major metabolite". Other reasons given were: "It is a unique human metabolite"; "Since virtually no metabolites were formed in rats"; "Since the metabolite was suspected to exhibit genotoxicity"; and "Reason unknown". In the studies investigated, no products were found to have toxicity associated with metabolites that greatly exceeded that of the parent compound. These findings provide further evidence that the number of products for which metabolites exhibit significantly higher toxicity than the parent compound is very small. Nevertheless, these data were obtained for approved drugs, and thus potentially hazardous products suspected of a high degree of metabolite toxicity may have been among the dropouts in the course of drug discovery and development. Future considerations of metabolite toxicity may have been necessary if these dropouts had continued in development.

Safety assessment of metabolites

The process of drug metabolism, excluding prodrugs, generally involves detoxification leading to reduction of pharmacological activity and elimination of the compound. Therefore, cases in which toxicities of a metabolite are completely different from those of the parent compound are believed to be rare. Furthermore, as shown by the examination of new drug approval data referred to above, very few products were noted to have unique human metabolites or to form metabolites that had significantly higher toxicity than the parent compound, thus it is believed that these cases are very rare. However, in some cases in clinical practice there have been concerns regarding manifestations of toxicity ascribable to metabolites, so safety assessment of metabolites found in humans appears to be necessary in these cases. In the FDA draft guidance, the rationale given for the 10-percent criterion in requiring safety assessment of metabolites is based on the amounts of metabolites formed with halothane, felbamate, cyclophosphamide, and acetaminophen; although other examples were provided in which signs and symptoms of toxicity were found with amounts of metabolites less than 10 percent. Accordingly, metabolites found only in humans should basically be subject to safety assessment using some type of method. Furthermore, since in some cases metabolites present in amounts less than 10 percent have been involved in manifestations of toxicity, investigations of the toxicity of these metabolites are called for. In this connection, we have investigated below cases in which toxicity studies of metabolites would be required.

1) Definition and method of assessment of metabolites

Phase I metabolites are formed through reactions

of oxidation, reduction, and hydrolysis, and Phase II metabolites through conjugation reactions. With the exclusion of *N*-acetylization, metabolism involves reactions leading to more polar compounds. Of the conjugate metabolites formed from Phase II reactions, glucuronide conjugates are generally less active than the parent compound. This is thought to be due to the significant molecular modification and increased polarization of aglycones, the decreased affinity of conjugates for receptors and enzymes due to steric hindrance, their reduced membrane permeability (tissue transferability), and the increased ease of their excretion (reduced exposure). It is therefore generally assumed that metabolite toxicity studies using conjugate metabolites are not required. However, cases exist in which there have been concerns over the formation of reactive metabolites in the form of acyl glucuronides or glutathione conjugates, or in which compounds such as irinotecan glucuronide conjugate are deconjugated by enteric bacteria in the intestinal tract after biliary excretion (Takasuna *et al.*, 1996). Thus, adverse reactions need to be fully identified in clinical studies.

Pharmacologically active metabolites are defined as circulating metabolites that have some pharmacological activity. In cases in which metabolites have pharmacological activity similar to or less than the parent compound, it is assumed that safety data have been obtained in the toxicity studies with the parent compound. However, in cases in which the pharmacological activity of the metabolite is greater than that of the parent compound, or in which the metabolite has pharmacological effects different from those of the parent compound, considerations in deciding the necessity for metabolite toxicity studies should include the exposure in humans and any differences between humans and animals in plasma concentration of the pharmacologically active metabolites. In addition, when adverse reactions are noted in clinical practice that cannot be predicted from the parent compound, or when there are strong concerns over the possibility of a relationship between such reactions and a metabolite, the assessment of metabolite toxicity may be needed. Valuable information is sometimes obtained from *in vitro* studies on receptors, enzyme inhibition, or non-clinical *in vivo* metabolite toxicity studies.

Reactive metabolites are intermediate metabolites formed in the process of metabolism that undergo covalent reactions with other physiological substances (proteins etc.). In the safety assessment of reactive metabolites, the investigations undertaken and inferences made from the study results should be based on the test systems in which the reactions occur *in vivo*. It may thus be of little use to determine toxicity through administration to laboratory animals of the final metabolite conjugate instead of the reactive metabolic intermediate. In addition, given the difficulty of chemically synthesizing reactive metabolites formed as metabolic intermediates, *in vitro* toxicity studies incorporating metabolic systems (S9 [9,000 × g supernatant fraction], microsomes, hepatocytes, liver slices) should be considered and studies conducted to appropriately evaluate potential toxicities of the metabolites. However, estimation of the relationships that may exist between clinical adverse events and reactive metabolites is by no means easy; thus, caution should be used when interpreting and extrapolating the *in vitro* findings to clinical findings. Further, consideration of the pathway by which metabolism and elimination occurs is also essential in assessing metabolites. For example, since metabolites excreted in urine are prone to being excreted with polar radicals attached, it is preferable to examine metabolite structure rather than amount of excretion.

Given the differences of opinion found in the public comments studied by the JPMA concerning the 10-percent criterion for safety testing of metabolites described in the FDA draft guidance, and the discrepancy in the value of the metabolite formation ratio established by PhRMA that would require metabolite safety assessment (FDA CDER, Guidance for Industry, Safety Testing of Drug Metabolites (Draft). 2005), it would appear that no clear and agreeable general criterion has been established. Further, studies on humans using radiolabeled forms (RI) are required to determine the metabolite formation, although there are no requirements for these studies in Japan, human RI data have been submitted for many of the products applied for recently. The studies provide important metabolite information used in determining whether or not to conduct animal studies on metabolites or to examine the toxicity of processes of metabolism as they relate to the causes of toxicity obtained from the overall assessment of adverse reactions, signs and symptoms of toxicity, and other manifestations in clinical studies.

2) Safety assessment in laboratory animals

In cases in which metabolite exposure has already been demonstrated in the toxicity studies with the parent compound, follow-up metabolite toxicity studies are usually considered unnecessary. However, in cases in which plasma metabolite exposure in animals is far less than in humans, the metabolite safety

cannot be assured from the results of studies using the dosages of parent included in the animal studies. Therefore, verification of safety is required when there are concerns over the toxic effects of metabolites. In some cases, metabolite exposure may be able to be increased by changing the dosage of the parent compound administered to animals, the number of doses, the rate of administration, or the route of administration.

Even when the safety of unique human metabolites cannot be verified in animal toxicity studies of the parent compound, the safety will at least be demonstrated in clinical studies for the duration and exposure used in the clinical study in which the metabolites were detected. When this occurs, the safety assessment of unique human metabolites will follow a different strategy from that of the parent compound. The chemical structure of unique human metabolites should be identified and the plasma concentration in humans determined. *In silico* determination of toxicity based on structure-activity correlations may also be useful at this stage. In addition, data on adverse reaction reports from clinical studies is essential, and these may lead to further metabolite safety studies.

While the findings of clinical studies are useful, they are limited to results from non-invasive examinations, whereas in animal studies a final necropsy can be performed, allowing tissue damage to be pathologically identified. Such animal studies are therefore essential, in that data unable to be obtained in humans can be obtained in the animals. When adverse events in humans are noted, it is essential in some cases to attempt to correlate these with the results of non-clinical studies. In addition, in many cases it is useful if scientifically valid metabolite toxicity studies can be conducted. When unique human metabolites exist, it may also be useful in some cases to compare the toxicity of the parent compound to that of the metabolite in single-dose, two-week or other toxicity studies.

3) Issues related to metabolites in clinical practice

Drugs given in low doses clinically are generally assumed to yield low metabolite exposure and correspondingly few concerns regarding manifestations of toxicity. Conversely, drugs given in high doses clinically require attention to the possibility of adverse reactions ascribable to metabolites.

In addition, humans are genetically diverse and exhibit individual variations in levels of expression of metabolic enzymes or in metabolic activity. Furthermore, various types of disorders (hepatic, renal, cardiac etc.) may lead to significant variability in pharmacokinetics from that of normal subjects resulting in variations in the parent compound or metabolite plasma concentrations. In addition, drug interactions may occur resulting in variations in the extent of metabolite exposure depending on other factors such as concomitant medications, diet, or lifestyle-related diseases.

In cases in which the metabolite of concern is formed by metabolic enzymes known to exhibit ethnic variations, differences in exposure to the parent compound as well as metabolites between Japanese and other subjects may exist. In such cases, caution is needed in extrapolating data from clinical studies conducted elsewhere to Japanese subjects.

Timing of non-clinical studies on metabolites

The prompt supply of high-quality drugs to the medical frontline is of crucial importance to patients. Non-clinical studies are of vital importance in ensuring the safe administration of drugs in clinical studies. To this end, the timing of non-clinical studies is essential in the process of drug development. Ideally, during the exploratory stage prior to the first administration to humans (First Human Dose and Phase I clinical studies), screening for metabolic stability and for pharmacologically active metabolites should take place, so that compounds selected will give little cause for concern regarding metabolite toxicity. In addition, *in vitro* metabolic studies on S9, microsomes, hepatocytes, liver slices, and other samples obtained from the liver of humans, mice, rats, dogs, monkeys, and other species will allow metabolic profiles to be identified, metabolic species differences to be investigated, and further metabolite characterization using LC-MS/MS, LC-NMR, and other techniques. Based on these results, the decision on which animal species to use in non-clinical safety studies can be determined by comparing the degree of similarity in human and animal metabolism (metabolite formation, metabolic rate). In addition, to avoid toxicity, it may also be useful to screen for reactive metabolites referred to above and to select compounds with minimal risk of formation of such metabolites.

Human metabolites formed *in vivo* can only be confirmed after administration of the drug to humans. In Phase I clinical studies, human samples (plasma, urine, feces) can be evaluated for the presence of metabolites and the metabolite's potential structure identified. Identifying the major human metabolites and assessing for the presence of any unique human

metabolites is of paramount importance. Once the potential metabolites are identified, then an estimation of their toxicity can be made by comparing the chemical structure of the metabolite with that of the parent compound. When appropriate, estimation of the changes in plasma concentrations of metabolites will also be essential. However, detection of changes in the metabolite concentration over time may require longer-term studies. A fully characterized synthesized reference standard of the identified metabolite will need to be provided, and further identification studies of metabolites separate from the clinical study may also need to be considered.

Studies using RI are useful for investigating the extent of metabolite exposure in humans. Microdosing (MD) studies using very low levels of RI may provide the ratio of the parent compound or metabolite to the total exposure in humans at an early date since the MD studies can often be conducted at an early stage of development. Use of MD studies facilitates the early detection of unique human metabolites or the need for further analyses of the metabolite concentrations, and results from these studies in conjunction with the TK analysis can provide useful information on the assessment of metabolite safety.

In cases in which there are strong concerns over the involvement of metabolites in toxicity in humans, it is necessary to carefully examine the findings available on metabolite safety prior to commencement of large-scale clinical studies (Phase III). The FDA guidance recommends that findings on the safety assessment of metabolites be submitted before commencing Phase III clinical studies, especially when unique human metabolites are found that present toxicological problems. The safety of these unique human metabolites in animals should be verified prior to Phase II clinical studies, and if possible, performance of human RI studies should also be considered early in development.

Prior to filing a new drug application (NDA), the metabolic enzymes and metabolic pathways of major human metabolites of a drug should be determined. The metabolite structure should be identified and the exposure in humans and animals should be determined using blood samples. The pharmacological activity of the metabolite should be assessed as well. Additionally, an understanding of whether or not the metabolites are unique to humans is essential and if so, then additional studies may be warranted.

After launch of a new drug, post-marketing surveillance studies should be carefully performed and are essential to verify drug efficacy and safety in daily medical care as they provide a means to collect information on the appropriate use of drugs unable to be obtained in clinical trials prior to marketing (Table 4). In particular, serious adverse events reported after a new drug launches may include those which non-clinical or clinical study results did not predict. In this connection, for adverse events found in clinical practice, it may be necessary to carefully examine unpublished results and results not available at the time of the application for causal links.

Table 4. Findings of post-marketing surveillance.

Safety and usefulness on the market
Long-term toxicity that affects individuals up to the next generation
Extremely rare, serious, or unpredictable adverse effects
Frequency of adverse effects (time course)
Identification of new types of efficacy
Off-label use
Overdose
Medical economics of drug treatment

Issues concerning methods of metabolite assessment

Studies will sometimes have to be conducted to assess metabolite safety. However, in some cases adequate exposure cannot be obtained even with administration of the metabolite to animals using the route of administration of the parent compound, or only transient exposure can be obtained with intravenous treatment. Exposure to metabolites may require significant modification through such methods as changing the route of administration, the dosage, or the number of administrations, etc. However, notwithstanding the apparent degree of exposure in the systemic circulation, changes in tissue transferability associated with such factors as plasma protein binding, membrane permeability, or affinity for transporters may reduce exposure in the target tissue, resulting in an underestimation of the toxicity. Moreover, the toxicity of reactive metabolites that give cause for concern may not be able to be studied by administering the conjugate metabolite to animals. There are also technical problems, in that chemical synthesis of reactive metabolites and glucuronide conjugates, etc. is often difficult and synthesized reference standards of metabolites are sometimes impossible to prepare. In view of the practical problems such as these, studies of the feasibility of the safety assessment of metabolites are required. In some cases, the profile of toxicity of a metabolite will have to

be determined from numerous factors, including *in silico* predictions from chemical structure databases or *in vitro* studies incorporating human metabolic systems. The safety assessment of metabolites requires consideration of complex factors, and even when safety testing of metabolites is deemed unnecessary, a full explanation of the rationale will need to be provided.

FUTURE DEVELOPMENT

In the interest of delivering high-quality drugs to patients as quickly as possible, safety assessment of metabolites was discussed at the kick-off meeting of the Drug Evaluation Forum, with reference to the views of clinicians and other academic representatives, in order to provide useful information for developers and reviewers. In the course of drug development, safety in humans is predicted through means such as metabolite profiling and pharmacological activity screening, clinical pharmacokinetic studies, human RI studies, in addition to the safety studies using metabolite reference standards which are conducted when deemed necessary. Although at present there are no guidelines for metabolite safety testing in Japan, 40 percent of the compounds in licensed products have undergone some form of safety testing of metabolites, and few metabolites unique to humans or with significant toxicity appear to have been found. However, these findings are based only on products approved as new drugs and do not include cases where development of a compound was discontinued. Indeed, it is likely that a large amount of valuable information on metabolites exists in the latter cases. Accordingly, with the cooperation of pharmaceutical companies, it is essential to collect and pool data on the toxicity of metabolites, especially negative data. Establishment of a standard database could be part of the consideration when drafting of guidelines, etc., and preferably a database that will facilitate worthwhile safety assessment. In Japan, as well, pharmaceutical companies would create databases, including negative data, based on consensus regarding their role in contributing to the needs of society through the prompt supply of safe and effective drugs to patients. In addition, on the regulatory sciences front, post-marketing surveillance enables prompt communication of findings to prescribers and patients, and it is also advisable to collect data on metabolites during clinical trials.

It is of vital importance to analyze metabolites using human samples obtained in clinical studies and to determine whether unique human metabolites are major metabolites. However, since these tasks may also constitute a rate-limiting factor in drug development, in some cases drug risk-benefit considerations may require selection of the optimal strategy through use of PMDA consultations or other services. In general, even when unique human metabolites or major metabolites are found in clinical studies, these studies are often single-dose, with smaller dosages than in non-clinical safety studies, or the safety investigations are based on short-term clinical studies with few reported adverse reactions. It therefore cannot always be assumed that the safety of metabolites in humans has been verified. Moreover, after approval, a marketed drug will be used by large numbers of patients and in some cases will be administered long-term. The principal importance of safety studies is identification of the toxicity profile and estimation of safety in humans after administration of high dosages for adequate periods of time. Further, since in the course of drug development the human metabolite profile is often identified around the conclusion of clinical Phase I studies, it is conceivable that development may be slowed when results on metabolites are obtained. On the other hand, MD studies may be helpful in metabolite profiling, and may accelerate assessment of human metabolites. In addition, reactive metabolites, even if a problem in clinical practice, are often unverifiable in ordinary toxicity studies. Provisions should be made for screening systems using human samples that may better evaluate the potential for reactive metabolites and allow a comparison of the findings on reactivity in humans to the findings in animals.

Clinically, a seemingly small finding with use of drugs in humans may have a large impact, so it is essential not to overlook problems that arise at the medical frontline. Future challenges will include how to reduce clinical risks based on considerations of whether clinical problems with less frequency can be detected in the non-clinical studies, or how clinical information should be used to impact basic research. Although compounds supplied to the medical frontline as drugs have been assessed for safety through toxicity studies, post-marketing clinical problems not observed during drug development may occur and may differ considerably from those found during daily usage by large populations. Therefore, feedback to drug researchers of findings from the medical frontline could, for example, be important information useful for improving drug development. Nevertheless, the incidence of actual problems in patients is very low, and these may be problems that cannot be detected by

the non-clinical studies. Accordingly, in the event of adverse effects, it is essential to act as quickly as possible to find ways to understand the mechanism behind the event.

In these terms, the drafting of strict guidelines on the safety assessment of metabolites presents considerable difficulties. It may be preferable to prepare flexible guidelines to speed the development of useful drugs. However, while such guidelines should be scientifically valid, it may be difficult to establish fixed criteria for assessment of the safety of metabolites. The safety assessment of metabolites cannot readily be based on a single theoretical framework, and basically a case-by-case approach is called for. However, some common understanding should be shared. To this end, it is useful to first prepare an outline concept paper. A collective viewpoint is also relevant to any discussion on the need for guidance, etc. A degree of flexibility may also be helpful for drug development and, in turn, society at large. The safety of metabolites should be assessed using scientifically sound methods and take into account signs, symptoms and adverse reactions that may be unpredictable in clinical studies on the parent compound, but may be related to the metabolites. In addition, elimination of scientifically meaningless studies for the safety assessment of metabolites is essential for the prompt supply of high-quality drugs to the medical frontline.

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REFERENCES

Baillie, T.A., Cayen, M.N., Fouda, H., Gerson, R.J., Green, J.D., Grossman, S.J., Klunk, L.J., LeBlanc, B., Perkins, D.G. and Shipley, L.A. (2002): Drug metabolites in safety testing. Toxicol. Appl. Pharmacol., **182**, 188-196.

Baillie, T.A., Cayen, M.N., Fouda, H., Gerson, R.J., Green, J.D., Grossman, S.J., Klunk, L.J., LeBlanc, B., Perkins, D.G. and Shipley, L.A. (2003): Reply. Toxicol. Appl. Pharmacol., **190**, 93-94.

CPMP/EWP/560/95, 17, December (1997): The note of guidance on investigation of drug interactions, Committee for Proprietary Medicinal Products.

Davis-Bruno, K. L. and Atrakchi, A. (2006): A regulatory perspective on issues and approaches in characterizing human metabolites. Chem. Res. Toxicol., **19**, 1561-1563.

FDA CDER Guidance for Industry, Published in Federal Register, March (2003): Guidance for Industry; Bioavailability and Bioequivalence Studies for Orally Administered Drug Products - General Considerations.

FDA CDER Guidance for Industry, Published in Federal Register, June (2005): Guidance for Industry, Safety Testing of Drug Metabolites (Draft).

FDA CDER Guidance for Industry, Published in Federal Register, September (2006): Guidance for Industry; Drug Interaction Studies – Study Design, Data Analysis, and Implications for Dosing and Labeling (Draft).

Guengerich, F.P. (2006): Safety Assessment of Stable Drug Metabolites. Chem. Res. Toxicol., **19**, 1559-1560.

Hastings, K.L., El-Hage, J., Jacobs, A., Leighton, J., Morse, D. and Osterberg, R.E. (2003): Letter to the Editor. Toxicol. Appl. Pharmacol., **190**, 91-92.

Humphreys, W.G. and Unger, S.E. (2006): Safety assessment of drug metabolites: Characterization of chemically stable metabolites. Chem. Res. Toxicol., **19**, 1564-1569.

Inoue, T., Harada, Y., Furuta, S., Kawamura, Y., Kurihara, A., Kurokawa, M., Nakazawa, T. and Sagami, F. (2005): The industry's concerns on the inquiries derived from the review of non-clinical studies - Analysis of questionnaire survey data by JPMA, Pharm. Regul. Sci, **36**, 388-397.

Iyakushin no. 496, Jun. 26th, 1998: Guidance for non-clinical Pharmacokinetic studies.

Lazarou, J., Pomeranz, B.H. and Corey, P.N. (1998): Incidence of adverse drug reactions in hospitalized patients: a meta-analysis of prospective studies. JAMA., **15**, 279, 1200-1205.

Ohno, Y. (1992): Toxicity testing: Regulatory Perspectives. Proceedings of the First International Conference on Harmonisation. 1991 Nov.; Brussels, Belgium. Ed D'Arcy PFD and Harron DWG. The Queen's University of Belfast. 186-188.

Prueksaritanont, T., Lin, J.H. and Baillie, T.A. (2006): Complicating factors in safety testing of drug metabolites: Kinetic differences between generated and preformed metabolites. Toxicol Appl Pharmacol., **217**, 143-152.

Smith, D.A. and Obach, R.S. (2005): Seeing through the mist: Abundance versus percentage. Commentary on metabolites in safety testing. Drug Metab Dispos., **33**, 1409-1417.

Smith, D.A. and Obach, R.S. (2006): Metabolites and safety: What are the concerns, and how should we address them? Chem. Res. Toxicol., Dec., **19**, 1570-1579.

Takasuna, K., Hagiwara, T., Hirohashi, M., Kato, M., Nomura, M., Nagai, E., Yokoi, T. and Kamataki, T. (1996): Involvement of β-glucuronidase in intestinal microflora in the intestinal toxicity of the antitumor camptothecin derivative irinotecan hydrochloride (CPT-11) in rats. Cancer Res., **56**, 3752-3757.

The Japanese Society for the Study of Xenobiotics (JSSX, November 29th - December 1st, 2006, Tokyo): 21st Annual Meeting Abstracts 171-177.

The Pharmaceutical Society of Japan (Jan. 13th, 2007, Tokyo): Kick-Off Meeting of Drug Evaluation Forum - Toward an Innovative Medicine: From Non-clinical to Clinical. Abstracts.

Toxicology Q&A (2003): (Question 16 : Toxicological evaluation of unique human metabolites), Pharm. Regul. Sci, **34**, 794-795.

Yakushin no. 526, March 28th, 1975: Points to Consider for Approval Application Data for New Manufacture or Import Drugs.

Yamada, H., Yamaguchi, J., Iida, I. and Okuyama, S. (2006): Idiosyncratic drug toxicity, Folia Pharmacol. Jpn., **127**, 473-480.

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A Reliable and Sensitive Immunoassay for the Determination of Crustacean Protein in Processed Foods

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Among food allergens, crustacea such as shrimps, crabs, and lobsters are a frequent cause of adverse food reactions in allergic patients. The major allergen has been identified as a muscular protein, tropomyosin. A novel sandwich enzyme-linked immunosorbent assay (ELISA) for the detection and quantification of crustacean protein in processed foods was developed using the sample dilution buffer that is added to porcine tropomyosin. The sandwich ELISA method was highly specific for the Decapoda group, apart from minor cross-reactivities to other crustacea and mollusks. The recovery ranged from 85 to 141%, while the intra- and interassay coefficients of variation were less than 2.8 and 8.4%, respectively.

KEYWORDS: Crustacea; food allergy; enzyme immunoassay; ELISA; tropomyosin

INTRODUCTION

In industrialized countries, food allergies have represented an important health problem in recent years, and it is estimated that approximately 8% of children and 2% of adults have some type of food allergy (*1, 2*). Burks et al. (*3*) estimated that approximately 120 deaths related to food allergies occur yearly in the United States. In Japan, the number of patients with food allergies, especially among young children, is increasing.

To prevent possible life-threatening reactions, the only effective treatment is to strictly avoid the consumption of these allergenic foods. However, various studies have shown that severe allergenic reactions can be caused by the accidental intake of food products containing allergenic materials (*4, 5*). Therefore, sufficient information regarding potentially allergenic ingredients in food products is necessary. The issue of a labeling system for allergenic ingredients in food products has been discussed by international organizations, such as the Codex Alimentarius Commission of the World Health Organization (WHO) and the Food Agriculture Organization (Codex 1998). In 1999, the Joint FAO/WHO Codex Alimentary Commission Session agreed to label eight kinds of foods that contain ingredients known to be allergens, including soybeans (FAO 1995, *6*). In Japan, the Ministry of Health, Labor, and Welfare (MLHW) has enforced a labeling system for allergenic food material since April 2002 to provide information about these foods to the allergic consumer. In this system, labeling for five food products, including eggs, milk, wheat, buckwheat, and peanuts, is mandatory and is recommended for 20 other food materials, such as soybeans and shrimp. In Japan, it became clear, based on epidemiological investigations, that the number of patients with a crustacean allergy such as to shrimp or crab has increased (*7, 8*).

In recommendations regarding labeling, Crustacea labeling would be particularly important because of the almost unlimited uses of Crustacea and because the number of patients with an allergy to Crustacea has been increasing, although the crustacean allergy is still less prevalent than the peanut allergy in the food-allergic population (*9, 10*). Crustacean allergic reactions may occur due to trace amounts of the crustacean protein, and anaphylaxis to Crustacea has been reported (*11, 12*).

In the present study, we developed a reliable sandwich enzyme-linked immunosorbent assay (ELISA) method with a high sensitivity for Crustacea. We showed that this detection method could be applicable to food-processing products and that the trace amount of Crustacea contained in commercial food products can be detected using the proposed sandwich ELISA method.

MATERIALS AND METHODS

Food Samples. The black tiger prawn (*Penaeus monodon*) was purchased from Intergrated Aquaculture Specialist, Inc. (Cebu, Philippines). The common Crustacea and mollusks, namely, northern shrimp (*Pandalus borealis*), Japanese spiny lobster (*Panulirus japonicus*),

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Caribbean spiny lobster (*Panulirus argus*), red king crab (*Paralithodes camtschatica*), swimming crab (*Portunus trituberculatus*), Japanese mantis shrimp (*Squilla oratoria*), euphausia (*Euphausia similis*), opossum shrimp (*Neomysis japonica*), acorn barnacle (*Balanus rostratus*), goose barnacle (*Pollicipes mitella*), common octopus (*Octopus vulgare*), giant octopus (*Paroctopus dofleini*), ocellated octopus (*Octopus ocellatus*), Japanese common squid (*Todardes pacificus*), spear squid (*Loligo kobiensis*), cuttlefish (*Sepia subaculeata*), common scallop (*Patinopecten yessoensis*), Japanese oyster (*Crassostrea gigas*), bloody clam (*Scapharca broughtonii*), blue mussel (*Mytilus edulis*), short-neck clam (*Tapes japonica*), common freshwater clam (*Corbicula leane*), Japanese hard clam (*Meretix lusoria*), Sakhalin surf clam (*Spisula sachalinensis*), horned turban (*Turbo cornutus*), Japanese abalone (*Haliotis discus*), and whelk (*Babylonia japonica*), and other ingredients and commercial processed foods were purchased at local stores in Japan.

Preparation of Model Processed Foods. The model processed foods were prepared according to a previously reported cooking procedure (*13*). Specifically, the freeze-dried black tiger prawn muscle powder was mixed with raw foods and then cooked to prepare the processed food items containing the shrimp protein at 10 μg/g as the final concentration. The following were prepared as the model processed foods.

Fish meat sausages were made of minced fish flesh (yellow goatfish, atka mackerel, and walleye pollack), lard, sugar, salt, ice water, and the spiking powder. Lard, salt, sugar, ice water, and the spiking powder were added to the minced fish flesh and thoroughly mixed. The mixture was ground using a small cutter, and the kneaded mixture was manually placed into the fish sausage casings. These were then heated at 121 °C for 15 min, cooled in flowing water for 5 min, and then placed in a refrigerator at 5 °C overnight.

The freeze-dried egg soup was made of egg, potato starch, milk sugar (lactose), salt, and the spiking powder. Eggs, potato starch, milk sugar, salt, and the spiking powder were thoroughly mixed. The mixture was dissolved in a plastic tray, frozen in a deep freezer at −80 °C, and then lyophilized for 20 h.

The chicken meatball was made of white meat of chicken, lard, potato starch, sugar, and the spiking powder. Lard, potato starch, sugar, and the spiking powder were added to ground white meat of chicken and thoroughly mixed. The mixture was ground using a small cutter, and the kneaded mixture was manually placed into casings. These were then preserved in a deep freezer at −20 °C.

Preparation of Black Tiger Prawn Protein Standards. A 0.1 g sample of the freeze-dried black tiger muscle powder was added to 20 mL of phosphate-buffered saline [10 mM Na-phosphate, 154 mM NaCl (pH 7.4)] containing 5 g/L sodium dodecyl sulfate (SDS), 20 mL/L β-mercaptoethanol, 10 μL/mL protease inhibitor cocktail, and 10 μL/mL 0.5 M ethylenediaminetetraacetic acid (Halt protease inhibitor cocktail kit; Pierce, Rockford, IL). The mixture was then shaken for 15 h at room temperature for extraction. After the extraction, the sample was centrifuged at 10000*g* for 30 min and the supernatant was filtered through a 0.8 μm microfilter paper (DISMIC-25cs; Advantec, Tokyo, Japan) to obtain the extract. The extract was then heated at 100 °C for 10 min. The obtained extracts were analyzed using a 2D Quant Protein Assay Kit (GE Healthcare UK Ltd. NA, England).

Purification of Black Tiger Prawn Tropomyosin (BTTM), Red King Crab, Swimming Crab, Japanese Oyster, Common Scallop, Japanese Common Squid, and Porcine Tropomyosin (PTM). The purification of the BTTM was carried out according to the methods reported by Nagpal et al. (*14*), Ishikawa et al. (*15*), and Miegel et al. (*16*), respectively. The black tiger prawn muscles (100 g) were homogenized with 200 mL of a solution containing 20 mM KCl, 1 mM $KHCO_3$, 0.1 mM $CaCl_2$, and 0.1 mM dithiothreitol (DTT). After centrifugation (3000*g* for 5 min at 4 °C), 200 mL of acetone was added to the precipitant. The suspension was filtered through cheesecloth, and the residue was then washed three times with 200 mL of acetone. Finally, the residue was allowed to dry at room temperature for 2–3 h. The dried powder was extracted overnight at room temperature with 200 mL of 25 mM Tris-HCl buffer (pH 8.0) containing 1 M KCl, 0.1 mM $CaCl_2$, and 1 mM DTT. After filtration through cheesecloth, the residue was once more extracted with 200 mL of 1 M KCl. The extracts were combined and cooled to 4 °C. Ammonium sulfate was added to produce an approximate 30% saturation. After 2 h, the solution was centrifuged (18000*g* for 60 min at 4 °C) and ammonium sulfate was then added to the supernatant (60% saturation). After 2 h, the solution was centrifuged and the precipitant was dissolved in 20 mL of 5 mM Tris-HCl (pH 7.5) containing 0.1 mM $CaCl_2$, and 0.1 mM DTT and was dialyzed overnight against 6 L of the same solution. The pH was then adjusted to 4.6 by the addition of HCl, and the tropomyosin precipitate was removed by centrifugation. The precipitate was dissolved in 25 mM Tris-HCl (pH 8.0) containing 1 M KCl, 0.1 mM $CaCl_2$, and 0.1 mM DTT and then chromatographed on a HiLoad Superdex 200 pg column (Φ26 mm × 600 mm; GE Healthcare UK Ltd.) equilibrated with the same buffer. Fractions of 5 mL were collected at a flow rate of 2.5 mL/min. The SDS-polyacrylamide gel electrophoresis analyses for all fractions were performed, and the fractions with the band corresponding to 37 kDa were combined (*17*). The combined fraction was then diluted with an equal volume of 0.2% trifluoroacetic acid and applied to reverse-phase high-performance liquid chromatography on a Wakosil-II 5C18 AR prep column (Φ10 mm × 250 mm; Wako Chemicals, Japan). The column was eluted at a constant flow rate of 2.5 mL/min by a gradient of acetonitrile in 0.1% trifluoroacetic acid. The tropomyosin-containing fractions were collected and lyophilized. The red king crab, swimming crab, Japanese oyster, common scallop, and Japanese common squid tropomyosins were obtained according to the purification procedure of BTTM. Tropomyosin derived from the porcine skeletal muscle (PTM) was obtained using the purification procedure of Greaser et al. (*18*) and Bailey et al. (*19*).

Production of Monoclonal Antibodies and Rabbit Polyclonal Antibodies to BTTM. The anti-BTTM monoclonal antibodies were generated at Nippon Biotest Laboratories, Inc. (Tokyo, Japan). For the production of the monoclonal antibodies against BTTM, female BALB/c mice were immunized with the purified BTTM. Fusion of the spleen cells was performed according to the method of Kohler and Milstein (*20*). The cell culture supernatants were screened for specific anti-BTTM antibodies by a direct ELISA with purified BTTM on a solid phase. The positive hybridomas were cloned and subcloned by limiting dilution. The positive hybridoma cells were intraperitoneally administered into BALB/c mice to induce the ascite tumors. The antibody was purified from the ascite fluid using a HyperD Protein A column (Bio Sepra Inc., Marlborough, MA). The specificity of the monoclonal antibodies was demonstrated by a direct ELISA method with purified black tiger prawn, red king crab, Japanese oyster, common scallop, and Japanese common squid tropomyosins. The polyclonal antibodies were generated at Medical and Biological Laboratories Co., Ltd. (Nagoya, Japan). The rabbit antiserum against BTTM was produced by immunization of New Zealand rabbits with purified BTTM in Freund's adjuvant. Injections were repeated six times at appropriate intervals (7 days). Whole blood was collected, and the serum was separated. The polyclonal antibodies were purified from the serum using a HiTrap Protein A HP column (GE Healthcare UK, Ltd.). The polyclonal antibodies were immunoabsorbed against Japanese common squid purified tropomyosin. The immunoabsorption was performed using the Japanese common squid tropomyosin-coupled column to removed further antibodies to molluskan protein. The specificity of the absorbed polyclonal antibodies was demonstrated by direct ELISA using the various purified tropomyosins.

Preparation of Sample Solution. The samples were treated with the Ace AM-4 homogenizer (Nissei, Tokyo, Japan) a few times for 30 s for homogeneity. Nineteen milliliters of 120 mM Tris-HCl (pH 7.4) containing 1 g/L bovine serum albumin (BSA), 0.5 mL/L Tween 20, 5 g/L SDS, and 20 mL/L β-mercaptoethanol (*21*) was added to 1 g of a homogenized sample, which was then shaken for 12 h at room temperature for extraction. After the extraction, the sample was centrifuged at 3000*g* for 20 min, and the supernatant was filtered through 5AB paper (Advantec) to obtain the extract.

Procedure of the Direct ELISA. Polystyrene 96 well microtiter plates (Nalge Nunc international, Rochester, NY) were coated overnight at 4 °C with 100 μL of purified tropomyosin (0.5 μg/mL) in coating buffer (50 mmol/L sodium carbonate, pH 9.6). The plates were then washed three times with Tris-buffered saline (TBS; 20 mmol/L Tris-HCl, pH 7.4, containing 154 mmol/L NaCl). The plates were blocked

for 1.5 h at 25 °C with TBS containing 10 g/L BSA, 30 g/L sucrose, and 0.5 mL/L ProClin 950 (Supelco, Bellefonte, PA). After the plates were washed six times with TBS containing 0.5 mL/L Tween 20 (TBS-T), diluted monoclonal antibodies or polyclonal antibodies were added to the wells and incubated at 25 °C for 1 h. After the wells were washed with TBS-T, 100 μL of horseradish peroxidase-labeled goat antimouse or antirabbit IgG serum was added to each well. After washing, 100 μL of the substrate solution containing 3,3′,5,5′-tetramethylbenzidine (SureBlue TMB Microwell Peroxidase Substrate; KPL, Gaithersburg, MD) was added to each well, and the plate was incubated at 25 °C for 20 min. The reaction was stopped by the addition of 1 mol/L sulfuric acid (100 μL/well). The plate was then read on a SPECTRAmax 250 microplate reader (Molecular Devices Corp., Menlo Park, CA) at the wavelength of 450 nm.

Procedure of the Sandwich ELISA. Polystyrene 96 well microtiter plates (Nalge Nunc international) were coated with 100 μL/well monoclonal antibodies (Mab #32, 10 μg/mL; and Mab #54, 20 μg/mL in 50 mmol/L sodium carbonate, pH 9.6) for 18 h at 4 °C. After they were washed three times with TBS, the plates were blocked for 1.5 h at 25 °C with TBS containing 10 g/L BSA, 30 g/L sucrose, and 0.5 mL/L ProClin 950. After the blocking buffer had been aspirated, the plates were dried in an incubator for 2.5 h at 30 °C, sealed in an aluminum-coated pack with drying agent (I.D. Sheet Desiccant; I.D., Tokyo, Japan), and stored at 4 °C until used. The food sample extracts were diluted 1:20 with the sample dilution buffer [TBS containing 2 g/L BSA, 0.02 g/L PTM, 0.5 mL/L Tween 20, and 0.5 mL/L ProClin 950]. The diluted sample or calibrator (100 μL) was added in triplicate to the coated wells, and the plates were then incubated for 90 min at 25 °C. After the plate had been washed four times with TBS-T, horseradish peroxidase-conjugated absorbed polyclonal antibodies (100 μL) were added to each well, and the plate was then incubated for 90 min at 25 °C. After another four washes with TBS-T, 100 μL of 3,3′,5,5′-tetramethylbenzidine solution (SureBlue Reserve TMB Microwell Peroxidase Substrate, KPL) as a substrate was added to each well, and the plate was incubated at 25 °C for 20 min. The reaction was stopped by the addition of 1 mol/L sulfuric acid (100 μL/well). The plate was then read using a SPECTRAmax 250 microplate reader at a wavelength of 450 nm. Standard curves were obtained by plotting the absorbance vs the logarithm of the analyte concentration.

Eleven crustacean protein extracts and two crustacean purified tropomyosins were tested in the concentration range from 0 ng/mL to 90 μg/mL. The concentration–response curves were obtained by plotting the absorbance vs the logarithm of the analyte concentration, and the curves were fitted to a four-parameter logistic equation, $y = \{(A - D)/[1 + (x/C)^B]\} + D$, where A is the maximum absorbance at infinite concentration, B is the curve slope at the inflection point, C is the concentration of the analyte giving 50% responses (RC_{50}), and D is the minimum absorbance for no analyte. The reactivity values were calculated as follows: reactivity % = [RC_{50} of black tiger prawn protein (or tropomyosin)/RC_{50} of target crustacean protein (or tropomyosin)]. Seventeen molluskan sample extracts were diluted 1: 20 with the sample dilution buffer containing PTM and analyzed using the sandwich ELISA method.

Evaluation of Assay Variation. For determination of the intra-assay precision, the mean coefficients of variation (CVs) were based on 10 replicates. The interassay precision was determined as the mean CVs on the basis of triplicate analyses on 10 different days. The limit of detection (LOD) for the sandwich ELISA was calculated as three times the standard deviation (SD) of the buffer blank mean value after 25 experiments. The limit of quantification (LOQ) was calculated as 10 times the SD of the buffer blank mean values after 25 experiments.

RESULTS

Construction of Sandwich ELISA. To evaluate the characteristics of the absorbed polyclonal antibodies and monoclonal antibodies to the BTTM, we tested the reactivity using a direct ELISA assay. We showed that the absorbed polyclonal antibody could be clearly detected for the crustacean tropomyosin but not for the molluskan tropomyosin. For the preparation of monoclonal antibodies to BTTM, nine monoclonal antibodies were obtained. Of these monoclonal antibodies, Mab #32 and #54 gave a satisfactory specificity and reactivity. In the examination of the different antibody combinations, using Mab #32 and #54 as the capture antibody and the HRP-conjugated absorbed polyclonal antibody as the detected antibody for the sandwich ELISA was found to provide the best results in terms of sensitivity and specificity to determine the total crustacean protein. However, as shown in **Figure 1**, a satisfactory dilution linearity could not be obtained when the dilution tests were performed using the tentatively constructed sandwich ELISA method and the three model processed foods. These results suggest that the food matrix could affect the dilution linearity in the tentatively constructed ELISA method. Therefore, to improve the dilution linearity, we attempted to add the PTM to the sample dilution buffer. As shown in **Figure 2**, the dilution linearity was satisfactorily improved by the addition of the PTM to the sample dilution buffer (r^2 = 0.996–0.999), confirming parallelism between the calibrators and the food samples. We statistically compared the two correlation coefficients of the dilution curves obtained using a sample dilution buffer containing PTM and those obtained without PTM for the assay of the model processed foods. A statistical test between the two correlation coefficients was performed using the Z-transformation test. P values of less than 0.05 were considered statistically significant. In the case of the chicken meatball, there was a statistically significant difference between the two correlation coefficients of the dilution curve (P = 0.036). In the case of

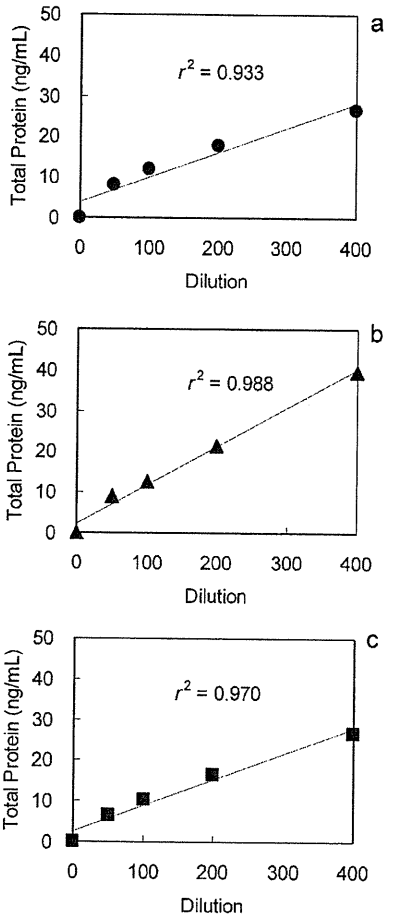

Figure 1. Linearity of dilution curves for model processed foods using the sample dilution buffer without PTM. (**a**) Fish meat sausage (solid circle); (**b**) freeze-dried egg soup (solid triangle); and (**c**) chicken meatball (solid square).

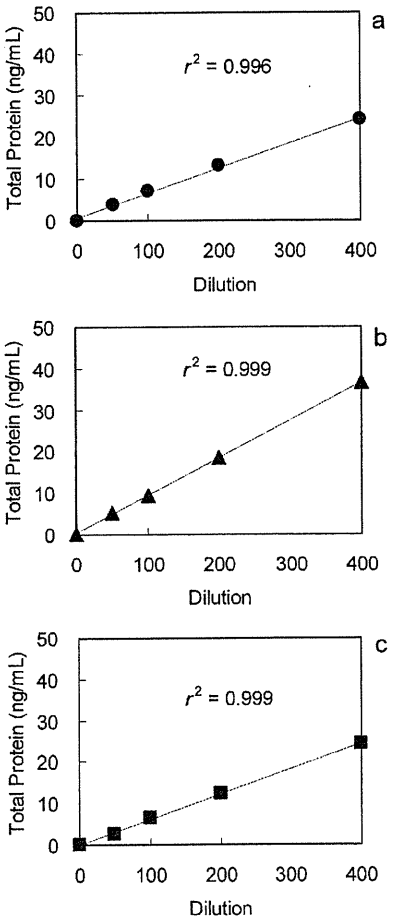

Figure 2. Linearity of dilution curves for model processed foods using the sample dilution buffer with PTM. (**a**) Fish meat sausage (solid circle); (**b**) freeze-dried egg soup (solid triangle); and (**c**) chicken meatball (solid square).

the fish meat sausage, a dilution curve using a sample dilution buffer to which was added PTM tended to show a good linearity when compared with using a tentative sample dilution buffer ($P = 0.081$). Meanwhile, there was no statistically significant difference in the freeze-dried egg soup ($P = 0.127$). These results suggested that the addition of PTM to the sample dilution buffer significantly improved the dilution linearity. Consequently, we established the sandwich ELISA method using a sample dilution buffer with PTM to minimize the food matrix effects.

Reactivity and Specificity Test. Various crustacean proteins, molluskan protein samples, and two crustacean purified tropomyosins (black tiger prawn and swimming crab) were examined to test the reactivity and specificity using the sandwich ELISA method. As shown in **Table 1**, the reactivities of the Decapoda group, which includes prawns and lobsters, are greater than 65.8%, and those of the crabs range between 28.5 and 38.5%. In contrast, the reactivities of the other Crustacea, such as the Japanese mantis shrimp, euphausia, and acorn barnacle, are less than 11.3%. The swimming crab purified tropomyosin demonstrated a reactivity of 154% as compared to the reactivity of black tiger purified tropomyosin. When all of the molluskan samples were tested, all of the levels were determined by the sandwich ELISA method to be less than 1.0 mg/kg (**Table 2**). These results suggest that the sandwich ELISA method has a specific reactivity to the Decapoda group, which includes prawns, shrimps, lobsters, and crabs.

Table 1. Reactivity Levels of Various Crustacean Samples in the Sandwich ELISA Method[a]

sample	RC_{50} (ng/mL)	reactivity (%)
Decapoda group		
black tiger prawn	9.5	
northern shrimp	14.4	65.8
Japanese spiny lobster	8.4	114.3
Caribbean spiny lobster	9.0	105.6
red king crab	24.6	38.5
swimming crab	33.4	28.5
other varieties of Crustacea		
Japanese mantis shrimp	124.4	7.6
euphausia	799.3	1.2
opossum shrimp	8060.4	0.1
acorn barnacle	83.8	11.3
goose barnacle	166.7	5.7

[a] RC_{50} is the concentration of analyte giving a 50% OD_{max} response. Reactivity % = (RC_{50} of black tiger prawn protein/RC_{50} of target crustacean protein).

Table 2. Cross-Reactivity of Various Molluskan Samples in the Sandwich ELISA Method

sample	cross-reactivity in ELISA (mg/kg)
Cephalopoda group	
common octopus	<1.0
giant octopus	<1.0
ocellated octopus	<1.0
Japanese common squid	<1.0
spear squid	<1.0
cuttlefish	<1.0
Bivalvia group	
common scallop	<1.0
Japanese oyster	<1.0
bloody clam	<1.0
blue mussel	<1.0
short-neck clam	<1.0
common freshwater clam	<1.0
Japanese hard clam	<1.0
Sakhalin surf clam	<1.0
Gastropoda group	
horned turban	<1.0
Japanese abalone	<1.0
whelk	<1.0

Limit of Detection and Limit of Quantification. The best model that describes the relationship between the absorbance and the antigen concentration is a four-parameter logistic curve (**Figure 3**). The LOD of the ELISA method determined using the standard proteins is 0.71 ng/mL, equivalent to 0.29 mg/kg samples, and LOQ is 2.25 ng/mL, equivalent to a 0.9 mg/kg sample. Consequently, the practical determination range lies between 1.56 and 50 ng/mL. For the final evaluation of the validation data for the sandwich ELISA and its application, the LOQ for routine analysis was set to 1.0 mg/kg sample. This level was considered to give a safety margin to the majority of consumers with an allergy to peanuts (22).

Quantification of Crustacean Protein in Model Processed Foods Using the Sandwich ELISA. To test the applicability of the sandwich ELISA in processed foods, the crustacean protein in three model processed food samples was determined using the sandwich ELISA. As shown in **Table 3**, the mean recoveries for all three model processed food samples ranged from 85 to 141%. The precision data from the three model processed foods are shown in **Table 4**. The interassay precision across all days was 5.3, 6.2, and 8.4% CV for the three model processed foods. The intra-assay precision for the three model processed foods was 2.8, 2.3, and 2.8% CV, respectively.

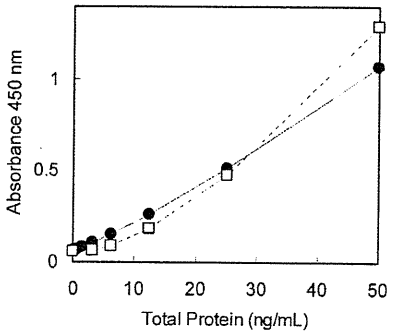

Figure 3. Representative standard curve using the shrimp protein standard in the sandwich ELISA method. The sample diluted buffer with PTM (solid circle); without PTM (open square).

Table 3. Recoveries of Crustacean Protein from Three Model Processed Foods

sample	concentration (mg/kg)	recovery (%)
fish meat sausage	25.0	96
	12.5	107
	6.3	114
	3.1	120
freeze-dried egg soup	29.8	124
	14.9	124
	7.4	125
	3.7	141
chicken meatball	25.0	97
	12.5	100
	6.3	105
	3.1	85

Table 4. Intra- and Interassay Variances in the Sandwich ELISA Method Using Three Model Processed Foods[a]

sample	concentration (mg/kg)	intra-assay	interassay
fish meat sausage	10	2.8	5.3
freeze-dried egg soup	11.9	2.3	6.2
chicken meatball	10	2.8	8.4

[a] The intra-assay variances were calculated from 10 replicates of the same extract, and the interassay variances were calculated from triplicate analysis of the same extract on 10 different days.

Application to the Commercial Food Products. Thirty-two different commercial food samples were analyzed by the sandwich ELISA method. Each commercial food was homogenized, and the extracts were obtained according to the extraction procedure described in the Materials and Methods section. As shown in **Table 5**, 15 commercial foods with a label of shrimp or crab on the ingredients list were clearly detected. In contrast, the levels in products without a label of shrimp or crab on the ingredients list were detected to be less than 1.0 mg/kg. There were no false positives from the no-declaration samples and no false negatives from the declaration samples analyzed in this study. When commercial food products containing shrimp or crab were serially diluted and assayed, each sample gave results close to linearity (r^2 = 0.993–1.000), confirming parallelism between the calibrators and the food samples. These results show that the sandwich ELISA method could appropriately determine the crustacean protein in the processed foods.

DISCUSSION

We established the sandwich ELISA method for the detection of crustacean protein that has a specific reactivity to the

Table 5. Analysis of Various Commercial Food Samples for Using the Sandwich ELISA Method

sample	declaration	substance	quantitative (mg/kg)	regression (r^2)
bean jammed	+	crab	264	0.998
seafood curry	+	shrimp	1780	0.999
beef curry	−		<1.0	
base of pilaf	+	crab	1100	1.000
cream pasta source	−		<1.0	
croquette	+	crab	404	1.000
croquette	−		<1.0	
croquette	−		<1.0	
dumpling	+	shrimp	77000	0.995
dumpling	+	crab	1040	1.000
dumpling	−		<1.0	
base of fried rice	+	shrimp	653	0.993
base of risotto	+	crab	36.7	0.998
spray-dried soup	−		<1.0	
gratin	+	shrimp	22400	0.995
gratin	−		<1.0	
snack	+	shrimp	100	0.998
cookie	−		<1.0	
Japanese rice cookie	−		<1.0	
fried food (prawn)	+	shrimp	282000	0.995
fried food (chicken)	−		<1.0	
fried food (poke)	−		<1.0	
fried food (oyster)	−		<1.0	
fried food (squid)	−		<1.0	
noodle	+	shrimp	145000	0.998
noodle	−		<1.0	
Japanese wheat noodle	−		<1.0	
steamed fish paste	+	crab	176	0.999
steamed fish paste	−		<1.0	
fried fish paste	+	shrimp	46.4	0.995
terrine	+	shrimp	1560	0.997
fish sausage	−		<1.0	

Decapoda group in Crustacea and applied this method to processed food. Jeoung et al. (*23*) already reported a determination method for tropomyosin. However, the cross-reactivity to mollusks and the application to processed foods have not yet been sufficiently clarified. Therefore, the reactivity and specificity of the sandwich ELISA method were tested using extracts from various Crustacea, mollusks, and commercial foods. In the test of all of the molluskan sample extracts, the reactivity levels were extremely low. The house dust mite was reported to cross-react with crustacean allergens (*24*). However, the monoclonal antibodies as the capture antibody do not cross-react with the house dust mite in the Western blot analysis (data not shown). These results suggest that this method would be specific to the Crustacea protein. However, the possibility of a cross-reaction with other less commonly used mollusks or other ingredients, such as crustacean extractants as seasonings, cannot be excluded and remains to be examined. It will be necessary to clarify the applicability of the present method.

The reactivities of lobster and prawn are similar to those of the black tiger prawn. Those of the crab group appear to be lower than those of the black tiger prawn. However, the purified swimming crab tropomyosin showed a high reactivity (154%). These results suggest that the variety of reactivities among the Decapoda group may be involved in the difference of the tropomyosin contents in the sample extracts.

Furthermore, we found that the addition of porcine skeletal tropomyosin to the sample dilution buffer in the sandwich ELISA method can appropriately determine the crustacean protein in processed foods without any food matrix effects.

As described in the Results section, the sample extracts of the model processed foods were serially diluted and assayed using the tentatively constructed sandwich ELISA method, and

a good linearity could not be observed (r^2 = 0.936–0.995). We considered that this result would be due to food matrix effects. Therefore, to improve the dilution linearity, we attempted to add the PTM to the sample dilution buffer. Consequently, the dilution linearity for model processed food was significantly improved by the addition of the PTM to the sample dilution buffer (r^2 = 0.996–0.999). The addition of troponin or actin failed to improve the dilution linearity (data not shown). These results suggest that tropomyosin may be involved in the food matrix effects, although the food matrix effect mechanism remains unclear. This method offers a new perspective for the determination of various proteins in processed food and is expected to be extremely useful in other protein-measuring methods using ELISA.

To evaluate the sandwich ELISA method for the determination of crustacean protein in processed foods, a recovery study and intra- and interassays were tested using model processed foods. The results of the analysis show that this method has a good accuracy and precision. The sandwich ELISA method's sensitivity was 0.71 ng/mL, corresponding to the 0.29 μg crustacean protein/g food sample weight. This result indicates that the sandwich ELISA method is suitable for detection in the presence of hidden crustacean protein in processed foods.

In conclusion, this sandwich ELISA method is shown to have an acceptable accuracy and precision and no false positive or false negative. This method has been demonstrated to be suitable for the quantitative measurement of the specific crustacean protein in processed foods without food matrix effects.

LITERATURE CITED

(1) Jansen, J. J.; Kardinaal, A. F.; Huijbers, G.; Vlieg-Boerstra, B. J.; Martens, B. P.; Ockhuizen, T. Prevalence of food allergy and intolerance in the adult Dutch population. *J. Allergy Clin. Immunol.* **1994**, *93*, 446–456.

(2) Bousquest, J.; Bjorksten, B.; Bruijnzeel-Koomen, C. A.; Hugget, A.; Ortolani, C.; Warner, J. O.; Smith, M. Scientific criteria and the selection of allergenic foods for product labeling. *Allergy* **1998**, *53*, 37–21.

(3) Burks, W.; Bannon, G. A.; Sicherer, S.; Sampson, H. A. Peanut-induced anaphylactic reactions. *Int. Arch. Allergy Immunol.* **1999**, *119*, 165–172.

(4) Bock, S. A.; Atkins, F. M. The natural history of peanut allergy. *J. Allergy Clin. Immunol.* **1989**, *83*, 900–904.

(5) Furlong, T. J.; DeSimone, J.; Sicherer, S. H. Peanut and tree nut allergic reactions in restaurants and other food establishments. *J. Allergy Clin. Immunol.* **2001**, *108*, 867–870.

(6) Hefle, S. L.; Nordlee, J. A.; Taylor, S. L. Allergenic foods. *Crit. Rev. Food Sci. Nutr.* **1996**, *36*, S69–89.

(7) Ebisawa, M.; Ikematsu, K.; Imai, T.; Tachimoto, H. Food allergy in Japan. *J. World Allergy Org.* **2003**, *15*, 214–217.

(8) Ebisawa, M. Epidemiology of food allergy (its comparison between Japan and other countries). *Arerugi* **2007**, *56*, 10–18.

(9) Bock, S. A.; Atkins, F. M. Patterns of food hypersensitivity during sixteen years of double-blind, placebo-controlled food challenges. *J. Pediatr.* **1990**, *117*, 561–567.

(10) Sicherer, S. H.; Furlong, T. J.; Maes, H. H.; Desnick, R. J.; Sampson, H. A.; Gelb, B. D. Genetics of peanut allergy: A twin study. *J. Allergy Clin. Immunol.* **2000**, *106*, 53–56.

(11) Sampson, H. A.; Metcalfe, D. D. Food allergies. *J. Am. Med. Assoc.* **1992**, *268*, 2840–2844.

(12) Foucard, T.; Malmheden, Y. I. A study on severe food reactions in Sweden—Is soy protein an underestimated cause of food anaphylaxis? *Allergy* **1999**, *54*, 261–265.

(13) Matsuda, R.; Yoshioka, Y.; Akiyama, H.; Aburatani, K.; Watanabe, Y.; Matsumoto, T.; Morishita, N.; Sato, H.; Mishima, T.; Gamo, R.; Kihira, Y.; Maitani, T. Interlaboratory evaluation of two enzyme-linked immunosorbent assay kits for the detection of egg, milk, wheat, buckwheat, and peanut in foods. *AOAC Int.* **2006**, *89*, 1600–1608.

(14) Nagpal, S.; Rajappa, L.; Metcalfe, D. D.; Subba Rao, P. V. Isolation and characterization of heat-stable allergens from shrimp (*Panaeus indicus*). *J. Allergy Clin. Immunol.* **1989**, *83*, 26–36.

(15) Ishikawa, M.; Shimakura, K.; Nagashima, Y.; Shiomi, K. Isolation and Properties of allergenic proteins in the Oyster *Crassostrea gigas*. *Fish. Sci.* **1997**, *64*, 610–614.

(16) Miegel, A.; Kobayashi, T.; Maeda, Y. Isolation, purification and partial characterization of tropomyosin and troponin subunits from the lobster tail muscle. *J. Muscle Res. Cell Motil.* **1992**, *13*, 608–618.

(17) Suma, Y.; Ishizaki, S.; Nagashima, Y.; Lu, Y.; Ushio, H.; Shiomi, K. Comparative analysis of barnacle tropomyosin: divergence from decapod tropomyosins and role as a potential allergen. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* **2007**, *147*, 230–236.

(18) Greaser, M. L.; Gergely, J. Reconstitution of troponin activity from three protein components. *J. Biol. Chem.* **1971**, *246*, 4226–4233.

(19) Bailey, K. Tropomyosin: A new asymmetric protein component of the muscle fibril. *Biochemistry* **1948**, *43*, 271–279.

(20) Kohler, G.; Milstein, C. Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. *Eur. J. Immunol.* **1976**, *6*, 511–519.

(21) Watanabe, Y.; Aburatani, K.; Mizumura, T.; Sakai, M.; Muraoka, S.; Mamegosi, S.; Honjoh, T. Novel ELISA for the detection of raw and processed egg using extraction buffer containing a surfactant and a reducing agent. *J. Immunol. Methods* **2005**, *300*, 115–123.

(22) Hourihain, J. O. B.; Kilburn, S. A.; Nordlee, J. A.; Hefle, S. L.; Taylor, S. L.; Warner, J. O. An evaluation of the sensitivity of subjects with peanut allergy to very low dose of peanut protein: A randomized, double-blind, placebo-controlled food challenge study. *J. Allergy Clin. Immunol.* **1997**, *100*, 596–600.

(23) Jeoung, B.; Reese, G.; Hauck, P.; Oliver, J. B.; Daul, C. B.; Leher, S. B. Quantification of the major brown shrimp allergen Pen a 1 (tropomyosin) by a monoclonal antibody-based sandwich ELISA. *J. Allergy Clin. Immunol.* **1997**, *100*, 229–234.

(24) Witteman, A. M.; Akkerdass, J. H.; Leeuwen, J.; Zee, J. S.; Aalberse, R. C. Identification of a cross-reactive allergen (presumably tropomyosin) in shrimp, mite and insects. *Int. Arch. Allergy Immunol.* **1994**, *105*, 56–61.

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Endocrine Disrupter Bisphenol A Increases In Situ Estrogen Production in the Mouse Urogenital Sinus[1]

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ABSTRACT

The balance between androgens and estrogens is very important in the development of the prostate, and even small changes in estrogen levels, including those of estrogen-mimicking chemicals, can lead to serious changes. Bisphenol A (BPA), an endocrine-disrupting chemical, is a well-known, ubiquitous, estrogenic chemical. To investigate the effects of fetal exposure to low-dose BPA on the development of the prostate, we examined alterations of the in situ sex steroid hormonal environment in the mouse urogenital sinus (UGS). In the BPA-treated UGS, estradiol (E_2) levels and CYP19A1 (cytochrome P450 aromatase) activity were significantly increased compared with those of the untreated and diethylstilbestrol (DES)-treated UGS. The mRNAs of steroidogenic enzymes, *Cyp19a1* and *Cyp11a1*, and the sex-determining gene, *Nr5a1*, were up-regulated specifically in the BPA-treated group. The up-regulation of mRNAs was observed in the mesenchymal component of the UGS as well as in the cerebellum, heart, kidney, and ovary but not in the testis. The number of aromatase-expressing mesenchymal cells in the BPA-treated UGS was approximately twice that in the untreated and DES-treated UGS. The up-regulation of *Esrrg* mRNA was observed in organs for which mRNAs of steroidogenic enzymes were also up-regulated. We demonstrate here that fetal exposure to low-dose BPA has the unique action of increasing in situ E_2 levels and CYP19A1 (aromatase) activity in the mouse UGS. Our data suggest that BPA might interact with in situ steroidogenesis by altering tissue components, such as the accumulation of aromatase-expressing mesenchymal cells, in particular organs.

aromatase, bisphenol A, developmental biology, embryo, estradiol/estrogen receptor, in situ estrogen production, male reproductive tract, prostate, steroidogenic enzyme, urogenital sinus

INTRODUCTION

Endocrine-disrupting chemicals (EDCs) have been implicated in the alteration of fetal development of urogenital organs as well as the reproductive and endocrine systems in humans and other species [1]. The fetal development of urogenital organs is induced by endogenous hormonal messages that originate in fetal and maternal hormone systems. Fetal exposure to EDCs disrupts the interactions between endogenous hormones and their receptors, causing adverse effects later in life [2]. In the prostate, both androgens and estrogens play a significant role in development and differentiation as well as in the maintenance of adult homeostasis [3]. Therefore, even small changes in estrogen levels, including those of estrogen-mimicking chemicals, can lead to changes in prostate development and differentiation.

Bisphenol A (BPA), one of the EDCs, is a well-known, ubiquitous, estrogenic chemical used in the manufacture of polycarbonate plastics, as a lining in metal food and drink cans, and in dental sealants [4]. The concern with BPA originates from its detection in maternal and fetal plasma as well as the placenta [5, 6]. Thus, fetal exposure to BPA is implicated in fetal toxicity as well as in subsequent growth of the infant. Histopathologically, fetal exposure to low-dose BPA (10 μg kg^{-1} day^{-1}) has been shown to increase cell proliferation of urogenital sinus epithelium (UGE) in the primary prostatic ducts of CD1 mice [7]. Recently, our group reported that fetal exposure to low-dose BPA (20 μg kg^{-1} day^{-1}) specifically increased the number of basal epithelial cells in the adult prostate of BALB/c mice and also induced permanent cytokeratin 10 expression in such cells similar to the effects of synthetic estrogen diethylstilbestrol (DES; 0.2 μg kg^{-1} day^{-1}) [8]. Epigenetically, neonatal exposure of male rats to low-dose BPA (10 μg kg^{-1} day^{-1}) elicited critical molecular changes during prostate development and also increased prostatic gland susceptibility to precancerous neoplastic lesions and hormonal carcinogenesis [9]. Toxicological studies of BPA at less than 50 μg kg^{-1} day^{-1} in rodent fetuses and offspring have demonstrated alterations of mammary gland development, open-field behavior, and reproductive functioning [10–12].

Some EDCs are reported to alter the in situ sex steroid hormonal environment in the reproductive system. The triazine herbicide atrazine binds directly to adrenal-4-binding protein/steroidogenic factor-1 (official symbol NR5A1) and increases CYP19A1 (cytochrome P450 aromatase) expression and, ultimately, estradiol (E_2) production in human genital cancer cell lines [13]. The aryl hydrocarbon (dioxin) also increases CYP19A1 (aromatase) expression mediated by its receptor in mouse ovaries [14]. In contrast, the phosphorothioate insecticide profenofos increases the expression of steroidogenic genes

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and testosterone levels in rat testes [15]. Recently reported adverse effects of BPA on in situ steroidogenesis include increased testosterone levels in mouse Leydig cells and decreased E_2 levels in porcine ovarian granulosa cells [16, 17]. Thus, BPA may have the potential not only to mimic estrogenic action but also to alter in situ steroidogenesis in the prostate as well as other reproductive organs.

To investigate the effects of fetal exposure to low-dose BPA on in situ steroidogenesis in the developing prostate, we first measured sex steroid hormone levels and CYP19A1 (aromatase) activity in the BPA-treated mouse urogenital sinus (UGS), from which the prostate develops embryologically. Subsequently, we examined the alterations of steroidogenic enzyme gene expression to confirm the alterations of the in situ sex steroid hormonal environment in the BPA-treated mouse UGS. Finally, we identified the BPA-specific biological effects for in situ steroidogenesis during fetal prostate development.

MATERIALS AND METHODS

Animals

In the present study, 36 pregnant female C57BL/6 mice were purchased on the 12th day of gestation from Japan SLC, where the breeding strategy was to mate three female C57BL/6 mice (age, 10 wk) with one male overnight and separate them the next morning (plug date denoted as Day 0). All animals were housed individually in chip-bedded polyolefin cages in a room with controlled temperature (23 ± 1°C) and humidity (45 to 65%) on a 12L:12D photoperiod. Mice were fed a low-phytoestrogen diet (NIH-07PLD; Oriental Yeast Co.) and tap water ad libitum.

Chemicals

For the present study, both BPA and DES with a purity of 99% or greater were purchased from Nacalai Tesque and Wako Pure Chemical Industries, respectively.

Fetal Exposure to Chemicals

We randomly assigned 36 pregnant female C57BL/6 mice to three different treatment groups: BPA (20 μg kg^{-1} day^{-1}, n = 12) or DES (0.2 μg kg^{-1} day^{-1}, n = 12), both of which were dissolved in tocopherol-stripped corn oil (MP Biomedical, Inc.), administered by oral gavages on Embryonic Day (E) 13 to E16 and the control group, in which pregnant mice were fed tocopherol-stripped corn oil (2 ml/kg, n = 12). Previously, our group reported that this protocol of fetal exposure to BPA and DES resulted in similar histopathological changes of adult prostate—that is, increased basal epithelial cell number and induction of cytokeratin 10, a classic marker associated with squamous differentiation, in such cells [8]. Our dose level of BPA for the present study was also based on reported results suggesting that BPA is less than 100-fold less potent than DES. The Mie University's Committee on Animal Investigation approved the experimental protocol.

Termination and UGS Dissection

Between E17 and Postnatal Day (P) 1, all animals were terminated by an overdose of isoflurane followed by cervical dislocation. For each of the three groups, from 15 to 18 fetuses (both male and female) from three pregnant mice were collected at E17, E18, P0, and P1. The bladder and urethra were removed and dissected to isolate the UGS, and then the five or six UGS obtained were pooled as one sample. Thus, the 15–18 UGS were divided into three samples at each time point. The UGS, cerebellum, heart, kidney, testis, and ovary were collected in RNAlater (Applied Biosystems).

To isolate pure UGS, other tissues, such as the bladder, urethra, wolffian duct, seminal vesicle, and mullerian duct, were removed from both the male and female urogenital tracts. The histopathology of the mouse UGS was then examined by hematoxylin-and-eosin staining.

Measurements of In Situ E_2 Levels and CYP19A1 (Aromatase) Activity in UGS

The E_2 levels and CYP19A1 (aromatase) activity in UGS were determined by liquid chromatography-tandem mass spectrometry [18] and a tritiated water release assay [19], respectively, which were made available by Aska Pharma Medical. Briefly, the organs were homogenized, and the extracts were applied to a C18 Amprep solid-phase column (Amersham Biosciences) to remove contaminating fats. The E_2 was then separated using a normal-phase high-performance liquid chromatography system (Jasco) with a silica gel column (Cosmosil 5SI; Nacalai Tesque), and 100 pg of isotope-labeled [$^{13}C_4$]E_2 were added to extracts. The evaporated extracts were reacted with 5% pentafluorobenzyl bromide/acetonitrile, under KOH/ethanol, for 1 h at 55°C. After evaporation, the products were reacted with 100 ml of picolinic acid solution (2% picolinic acid, 2% 2-dimethylaminopyridinc, and 1% 2-methyl-6-nitrobenzoic acid in tetrahydrofuran) and 20 ml of triethylamine for 0.5 h at room temperature. The reaction products were dissolved in 1% acetic acid and then purified using a Bond Elute C18 column (Varian). The products were measured with a reverse-phase liquid chromatograph (Agilent 1100; Agilent Technologies) coupled with an API 5000 triple-stage quadrupole mass spectrometer (Applied Biosystems) in the positive-ion mode. This device monitored the *m/z* 558 to *m/z* 339 (E_2) and *m/z* 562 to *m/z* 343 ([$^{13}C_4$]E_2) transitions.

The tritiated water release assay was used for the measurement of CYP19A1 (aromatase) activity. This method measures the production of ^3H_2O, which forms as a result of aromatization of the substrate [1b-^{3}H]androst-4-ene-3,17-dione (New England Nuclear). Serum-free medium containing [1b-^{3}H]androst-4-ene-3,17-dione solution (54 nM) was prepared, of which 0.5 ml was added to each sample. After incubation for 1 h, the samples were placed on ice, and 200 μl of culture medium were withdrawn. The medium was extracted with 500 μl of chloroform, vortexed, and then centrifuged for 1 min at 9000 × *g*. A 100-μl aliquot of the aqueous phase was mixed with 100 μl of a 5% (wt/vol) charcoal/0.5% (wt/vol) dextran T-70 suspension, vortexed, and then incubated at room temperature for 10 min. Then, after centrifugation of the solution for 5 min at 9000 × *g*, a 150-μl aliquot was removed for measurement of radioactivity by liquid scintillation.

RNA Extraction and cDNA Preparation

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Inc.) in accordance with the manufacturer's instructions. The RNA concentration was then determined spectrophotometrically by a multidetection microplate reader (Dainippon Sumitomo Pharma Co.). From 50 ng of total RNA, cDNA was reverse transcribed using oligo(dT) and Superscript II RNase H-reverse transcriptase (Invitrogen) as previously described [8].

Analysis of Gene Expression Profile

For determining gene expression profiles of the male UGS, GeneChip analysis with the Percellome method was performed [20]. Briefly, organs were prepared using RLT buffer (Qiagen, Inc.). Total RNA was extracted using RNeasy Mini Kit. First-strand cDNA was synthesized by incubating 5 mg of total RNA with a T7 oligo(dT) primer (Invitrogen) according to the manufacturer's protocol. The dsDNA was mixed with T7 RNA polymerase (Enzo Biochem, Inc.). During the in vitro transcription, generated cRNAs were labeled with biotin-16-UTP and biotin-11-CTP (Enzo Biochem, Inc.). The purified cRNA was fragmented at 300–500 bp into the target solution. Hybridization was performed with the GeneChip Mouse Genome 430 Version 2.0 (Affymetrix, Inc.) at 45°C for 18 h after staining with streptavidin-R-phycoerythrin conjugates (Molecular Probes, Invitrogen). The reacted arrays were then scanned as digital image files, and the scanned data were analyzed with GeneChip Operating Software (Affymetrix, Inc.). The expression data were converted to copy numbers of mRNA per cell by the Percellome method, quality controlled, and analyzed using Percellome software [20].

Real-Time PCR Analysis

Real-time PCR was carried out in the iCycler iQ Detection System (Bio-Rad Laboratories) with iQ SYBR-Green Supermix reagents (Bio-Rad Laboratories) as previously described [8]. The PCR amplification reaction was performed with specific primers as shown in Table 1. After PCR, melting-curve analysis was performed to verify specificity and identity of the PCR products. All data were analyzed with the iCycler iQ Optical System Software Version 3.0A (Bio-Rad Laboratories). All PCR data were normalized to *Gapdh* mRNA.

Preparation of Primary Cultured Mesenchymal Cells from UGS

The UGS were dissected from the fetuses and separated into UGE and urogenital sinus mesenchyme (UGM) by tryptic digestion and mechanical separation as previously described [21]. UGM were cultured in RPMI-1640

TABLE 1. Sequences of oligonucleotide primers used for the real-time PCR analyses.

Gene	Primer[a]
Gapdh	F: 5′-AAATGGTGAAGGTCGGTGTG-3′
	R: 5′-TGAAGGGGTCGTTGATGG-3′
Cyp19a1	F: 5′-GCCCAATGAATTTACCCTCGAA-3′
	R: 5′-AAGCCAAAAGGCTGAAAGTACCT-3′
Cyp11a1	F: 5′-TCGACTCCTCAGAACTAAGACCTG-3′
	R: 5′-GTACCCTGGTGTCCTTTATAGCCT-3′
Nr5a1	F: 5′-CCTGGGCTGGCTACCTCTATC-3′
	R: 5′-CGAACTAGAGCCAGAGGAGGAC-3′
Esr1	F: 5′-GCACAGGATGCTAGCCTTGTCTC-3′
	R: 5′-AATTGTCACCAGCTTGCAGGTTC-3′
Ar	F: 5′-GGCGGTCCTTCACTAATGTCAACT-3′
	R: 5′-CTGACTTGTGCATGCGGTACTCAT-3′
Esrrg	F: 5′-CCGAGAGTTGGTGGTTATCATTGG-3′
	R: 5′-GGAAGACCCTCGCCGTGC-3′

[a] F, forward; R, reverse.

with 5% fetal bovine serum and plated out on four-well glass slides (BD Falcon). After several days, cells were fixed in methanol and processed for immunocytochemical analysis.

Immunocytochemical Staining

The sections were first incubated for 15 min in 0.01 M PBS. After inhibition of endogenous peroxidases (10 min in 0.6% H_2O_2 diluted in 0.01 M PBS plus 0.2% Triton X-100 [PBST]) and saturation (2 h in a 5% normal goat serum solution), sections were incubated overnight at 4°C in a polyclonal affinity-purified antiaromatase antibody or estrogen-related receptor gamma (ESRRG) antibody raised in rabbit against quail recombinant aromatase or ESRRG diluted 1:500 in 0.01 M PBST. The next day, the sections were immersed for 2 h at room temperature in a biotin-conjugated goat anti-rabbit immunoglobulin G (DakoCytomation, Inc.) diluted 1:400 in PBST and then for 2 h in a streptavidin-fluorescein complex (Rhodamine; DakoCytomation, Inc.) diluted 1:50 in PBST. Between each step, sections were extensively rinsed in PBST. The sections were mounted onto microscope slides, coverslipped with a gelatin-based mounting medium, and stored in the dark at 4°C. For double-labeling immunofluorescence, Alexa Fluor 488- or 594-conjugated secondary antibodies were used. Rabbit polyclonal anti-aromatase antibody was kindly provided by Prof. Nobuhiro Harada (Department of Biochemistry, Fujita Health University School of Medicine, Aichi, Japan) [22]. The rabbit polyclonal anti-ESRRG antibody used in the present study was established and characterized as previously reported [23]. The mouse monoclonal anti-Ran antibody (Santa Cruz Biotechnology, Inc.) was used to detect nucleus in cells. Ran, also called TC4, is the small RAS-related protein that is localized in the nucleus.

Statistical Analysis

Results are expressed as the mean ± SD. Differences among the three groups were determined using Student *t*-test with Dunnett multiple comparison. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

BPA-Specific Increases of E_2 Levels and CYP19A1 (Aromatase) Activity in Mouse UGS

The pregnant mice were exposed to low-dose BPA during the onset of prostatic budding (E13–E16), and the UGS of fetuses were collected during bud elongation (E17–P1). In analyses of in situ sex steroid hormonal environment, E_2 levels and CYP19A1 (aromatase) activity were significantly increased only at P1 in BPA-treated UGS, not at P1 in the DES-treated UGS (Fig. 1). At E17 and P1, both the E_2 levels and CYP19A1 (aromatase) activity in untreated male UGS were not significantly different compared with those in untreated female UGS.

BPA-Specific Up-Regulation of Steroidogenic Enzyme and Sex-Determining Gene mRNA in Mouse UGS

To investigate the BPA-specific gene alterations related to increases of the E_2 levels and aromatase activity, we performed preliminary GeneChip analysis with the Percellome method in the BPA- or DES-treated male UGS at E17 and P1. The results showed BPA-specific mRNA up-regulation of steroidogenic enzymes, such as *Cyp11a1*, *Cyp11b1*, and *Cyp17a1*, and sex-determining factors, such as *Nr5a1*, *Nr0b1*, *Gata4*, and *Amhr2* (data not shown). Furthermore, quantitative PCR analysis confirmed the mRNA up-regulation of *Cyp19a1*, *Cyp11a1*, and *Nr5a1* only in the BPA-treated neonatal (P0 and P1) UGS, not in the DES-treated neonatal UGS (Fig. 2). No difference in mRNA expression levels was found between E17 and P1 when comparing the untreated male UGS to that of the female. In

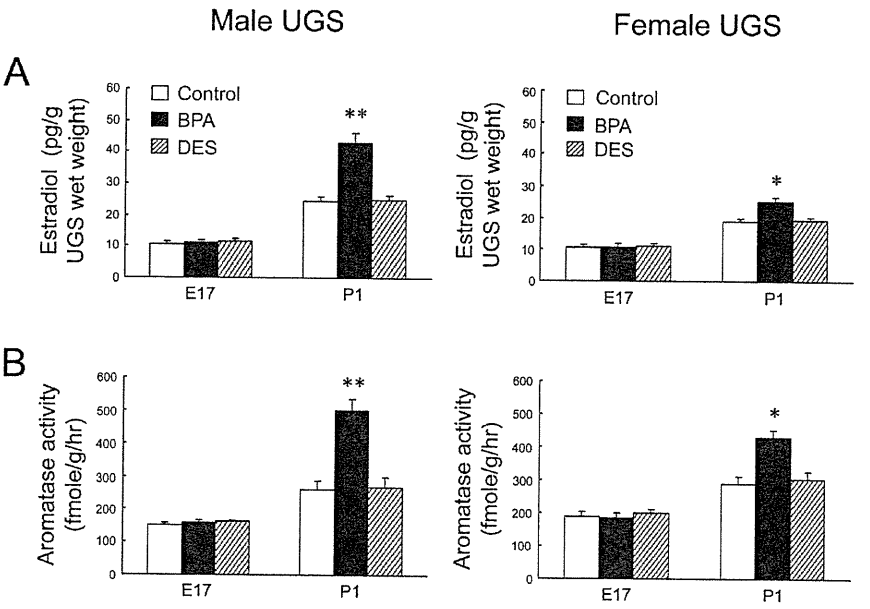

FIG. 1. BPA-specific increases of E_2 levels and CYP19A1 (aromatase) activity in mouse UGS. E_2 levels (**A**) and CYP19A1 (aromatase) activity (**B**) were measured in the untreated control (open bar), BPA-treated UGS (closed bar), and DES-treated UGS (slashed bar) at E17 and P1. $*P < 0.01$, $**P < 0.001$ vs. control.

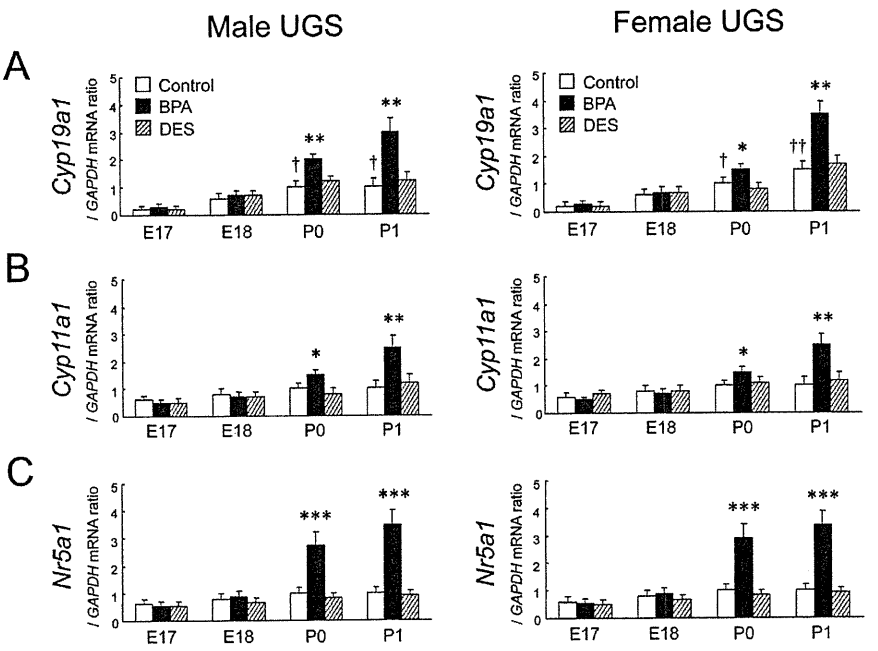

FIG. 2. BPA-specific up-regulation of steroidogenic enzyme and sex-determining gene mRNA in mouse UGS. The relative mRNA expressions of *Cyp19a1* (**A**), *Cyp11a1* (**B**), and *Nr5a1* (**C**) were determined in the untreated control (open bar), BPA-treated UGA (closed bar), and DES-treated UGS (slashed bar) between E17 and P1. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control at each time point; $^{\dagger}P < 0.01$, $^{\dagger\dagger}P < 0.001$ vs. control at E17.

untreated male and female UGS, the mRNA of *Cyp19a1* was gradually increased between E17 and P1.

Restricted BPA-Specific Up-Regulation of Steroidogenic Enzyme and Sex-Determining Gene mRNA in UGE and UGM

In male fetuses at P1, it was not feasible to separate UGE and UGM components within the male UGS because of the formation of prostatic buds. In the female at P1, the up-regulation of *Cyp19a1*, *Cyp11a1*, and *Nr5a1* mRNA was observed only in UGM, not in UGE, of the BPA-treated group (Fig. 3). In both male and female UGE, expressions of such mRNAs were quite low and not up-regulated, even in the BPA-treated group. At E17, no difference in mRNA expression levels was found when comparing the untreated male UGM with that of the female.

BPA-Specific Increases of Aromatase-Expressing Cells in Primary Cultured UGM

In both the male and female, P1 UGM was primary cultured in vitro. Representative pictures of aromatase-positive cells are shown in Figure 4, A–C. The aromatase-positive staining was

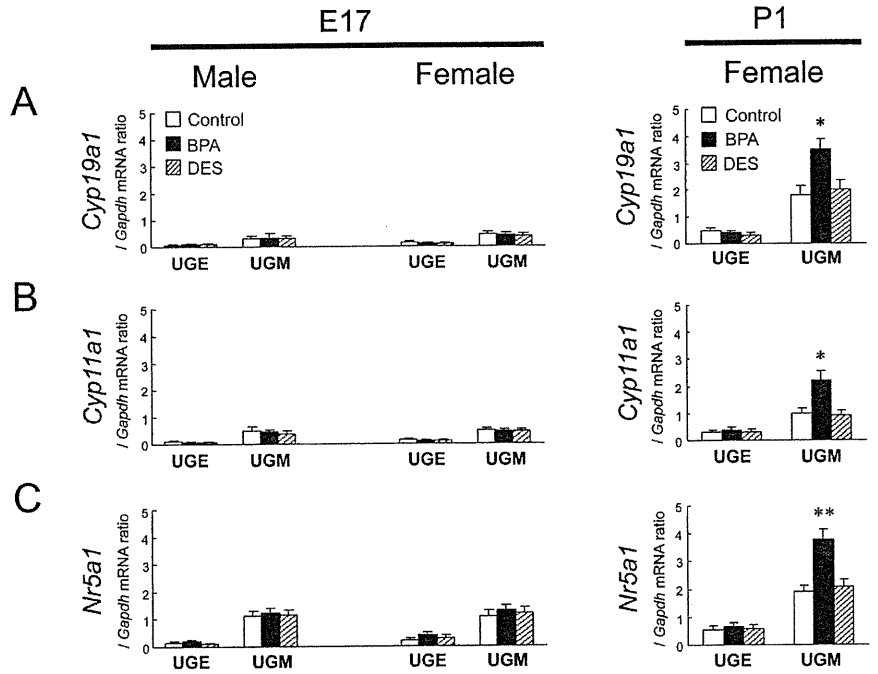

FIG. 3. Restricted BPA-specific up-regulation of steroidogenic enzyme and sex-determining gene mRNA in UGE and UGM. The relative mRNA expressions of *Cyp19a1* (**A**), *Cyp11a1* (**B**), and *Nr5a1* (**C**) were determined for UGE and UGM of the untreated control (open bar), BPA-treated UGS (closed bar), and DES-treated UGS (slashed bar) at E17 and P1. $^{*}P < 0.01$, $^{**}P < 0.001$ vs. control.

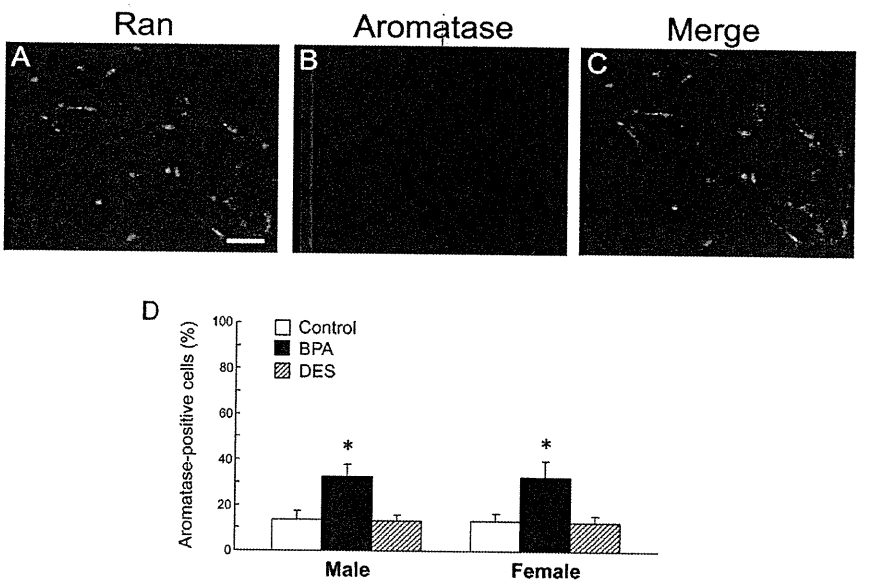

FIG. 4. BPA-specific increases of aromatase-expressing cells in primary cultured UGM. **A–C**) Fluorescence signals were detected for the CYP19A1 (aromatase) protein in primary cultured UGM. The nuclei were identified by Ran staining. Bar = 100 μm, magnification ×400. **D**) The number of aromatase-positive cells was counted in primary cultured UGM of the untreated control (open bar), BPA-treated UGS (closed bar), and DES-treated UGS (slashed bar), and the percentage of aromatase-positive cells was calculated from at least 10 areas. $*P < 0.01$ vs. control.

observed in the cytoplasm of cultured UGM. The rate of positivity (i.e., the percentage of cells that expressed CYP19A1 [aromatase] protein), was approximately 10% in the untreated and the DES-treated groups, whereas it was as high as approximately 30% in the BPA-treated group (Fig. 4D). No difference in the rate of positivity of CYP19A1 (aromatase) was found when comparing the untreated male UGM to that of the female.

Restricted BPA-Specific Up-Regulation of Esrrg *mRNA in UGE and UGM*

In E17 female UGM, the mRNA expression of *Esr1* was up-regulated by both BPA and DES treatment (Fig. 5A). At E17, however, the mRNA expression of *Ar* was up-regulated by both BPA and DES treatment in the male UGS (Fig. 5B). At P1, mRNA expression of *Ar* was up-regulated by both BPA and DES treatment in the female UGS (Fig. 5B). In both the male and female, the up-regulation of *Esrrg* mRNA was observed at E17 and restricted in UGM, but not in UGE, of the BPA-treated group (Fig. 5C). In both the male and female UGE, the expression of *Esrrg* mRNA was quite low and not up-regulated, even in the BPA-treated group. At E17, no difference in mRNA expression levels was found when comparing the untreated male UGS with that of the female.

BPA-Specific Increases of ESRRG-Expressing Cells in Primary Cultured UGM

In both the male and female, E17 UGM was primary cultured in vitro. Representative pictures of ESRRG-positive

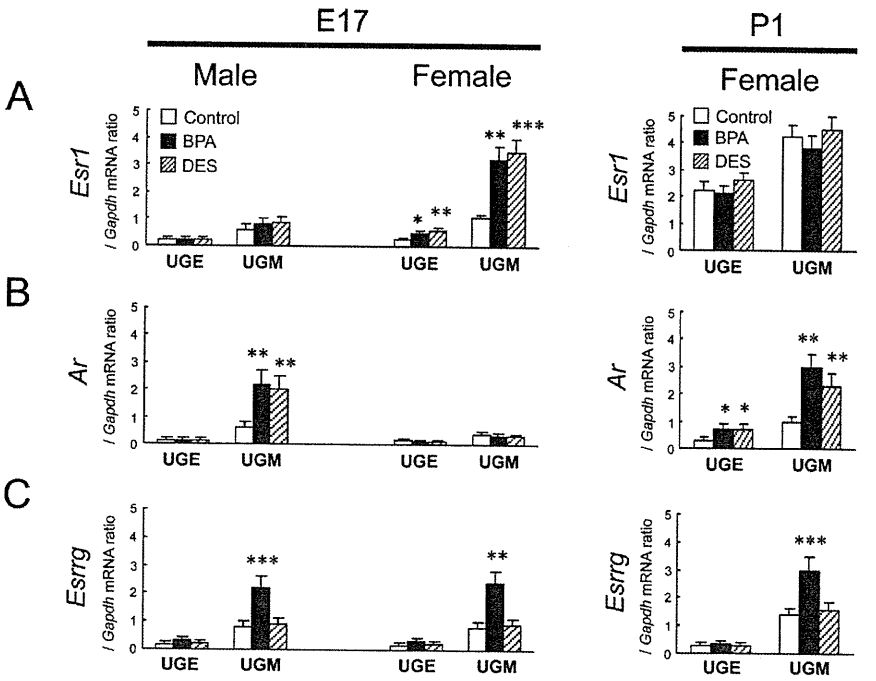

FIG. 5. Restricted BPA-specific up-regulation of *Esrrg* mRNA in UGE and UGM. The relative mRNA expressions of *Esr1* (**A**), *Ar* (**B**), and *Esrrg* (**C**) were determined in UGE and UGM of the untreated control (open bar), BPA-treated UGS (closed bar), and DES-treated UGS (slashed bar) at E17 and P1. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. control.

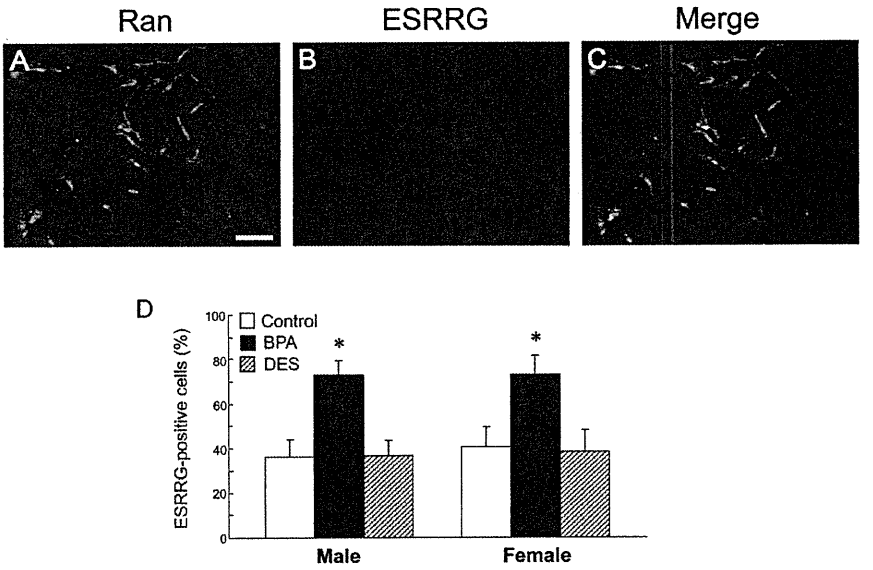

FIG. 6. BPA-specific increases of ESRRG-expressing cells in primary cultured UGM. **A–C**) Fluorescence signals were detected for the ESRRG protein in primary cultured UGM. The nuclei were identified by Ran staining. Bar = 100 μm, magnification ×400. **D**) The number of ESRRG-positive cells was counted in primary cultured UGM of the untreated control (open bar), BPA-treated UGS (closed bar), and DES-treated UGS (slashed bar), and the percentage of ESRRG-positive cells was calculated from at least 10 areas. *$P < 0.01$ vs. control.

cells are shown in Figure 6, A–C. The ESRRG-positive staining was observed in both the nucleus and the cytoplasm of cultured UGM. The number of ESRRG-positive UGM was significantly increased only in the BPA-treated group and showed a 2.2-fold increase in males and a 1.6-fold increase in females (Fig. 6D). No difference was found in the rate of positivity of ESRRG when comparing the untreated male UGM with that of the female.

BPA-Specific Up-Regulation of Esrrg *and Steroidogenic Enzyme mRNA in Sex Hormone-Related Organs*

To investigate the BPA-specific up-regulation of in situ steroidogenesis in other organs, we first examined the changes in *Esrrg* mRNA expression in sex hormone-related organs, such as the cerebellum, heart, kidney, ovary, and testis. At P1, the mRNA expression of *Esr1* in the cerebellum, heart, kidney, and ovary, but not in the testis, was up-regulated by both BPA and DES treatment (Fig. 7A). However, no significant difference in *Ar* mRNA expression was observed in all organs examined (Fig. 7B). In the untreated group, the mRNA expression of *Esrrg* was not detected in the testis at E17 and P1 (Fig. 7C). The up-regulation of *Esrrg* mRNA was observed at E17 and restricted to the cerebellum, heart, kidney, and ovary (Fig. 7C). The BPA-specific up-regulation of *Cyp19a1*, *Cyp11a1*, and *Nr5a1* mRNA was observed only at P1 in the cerebellum, heart, kidney, and ovary, but not in the testis (Fig. 8).

DISCUSSION

Concern about the effects of EDCs such as BPA on human health has been increasing [24]. Although the majority of EDCs have the potential to alter functioning of the reproductive and endocrine system, the actual mechanism responsible for such alterations has not been identified thoroughly. BPA is of concern because its chemical structure resembles that of DES. Several studies have reported that BPA can mimic estrogen action, such as induction of vaginal cornification, uterine vascular permeability, growth and differentiation of the mammary gland, and synaptic plasticity in the hippocampus [25–28]. In the prostate, alterations in normal development can produce permanent changes that persist throughout adulthood and may increase the risk of disease in later life [9]. Thus, our objective was to investigate the biological effects of low-dose BPA on the initial development of primary ducts in the fetal prostate.

During prostatic development, alteration of sex steroid hormone synthesis may be responsible for prostatic anomalies associated with fetal exposure to EDCs. In the present study, fetal exposure to low-dose BPA increased E_2 levels in P1 UGS of both the male and female, whereas DES-induced changes were not detected. This alteration was also correlated with increased activity of CYP19A1 (aromatase) in UGS at P1, suggesting the unique action of BPA for in situ steroidogenesis in UGS. The BPA-specific increase of E_2 levels in UGS at P1 was correlated with the following: mRNA up-regulation of steroidogenic enzymes, such as *Cyp19a1* and *Cyp11a1*, and an increased number of aromatase-expressing UGM. The enzyme CYP19A1 (aromatase) is responsible for in situ E_2 production and the crucial testosterone/E_2 balance necessary for normal embryonic and fetal development, even in males. The data presented here shows that the up-regulation of *Cyp19a1* mRNA in BPA-treated UGM was comparable to changes in both in situ E_2 production and CYP19A1 (aromatase) activity.

In the present study, we demonstrated that the BPA-specific increase in steroidogenic enzyme mRNA and aromatase-expressing cell number were observed in both the male and female UGM. During embryonic development, the mesenchymal component is involved in the induction and organogenesis of various organs, including the prostate, mammary gland, lung, kidney, and pancreas. It has been well established that subpopulations of the mesenchymal component are a source of potent molecules that regulate epithelial growth and differentiation [29]. In the prostate, androgen-responsive signals derived from UGM permissively and instructively induce UGE to form primary ducts of the prostate [30].

Comparison between the neonatal male and female UGS shows a similarity in the condensed mesenchyme of the ventral areas—that is, the ventral prostate mesenchyme (VPM) in the male and the ventral mesenchymal pad (VMP) in the female [31]. In the male, a defined VPM is specifically associated with ductal branching morphogenesis and cytodifferentiation of the ventral prostate. Females do not usually form a prostate. In a

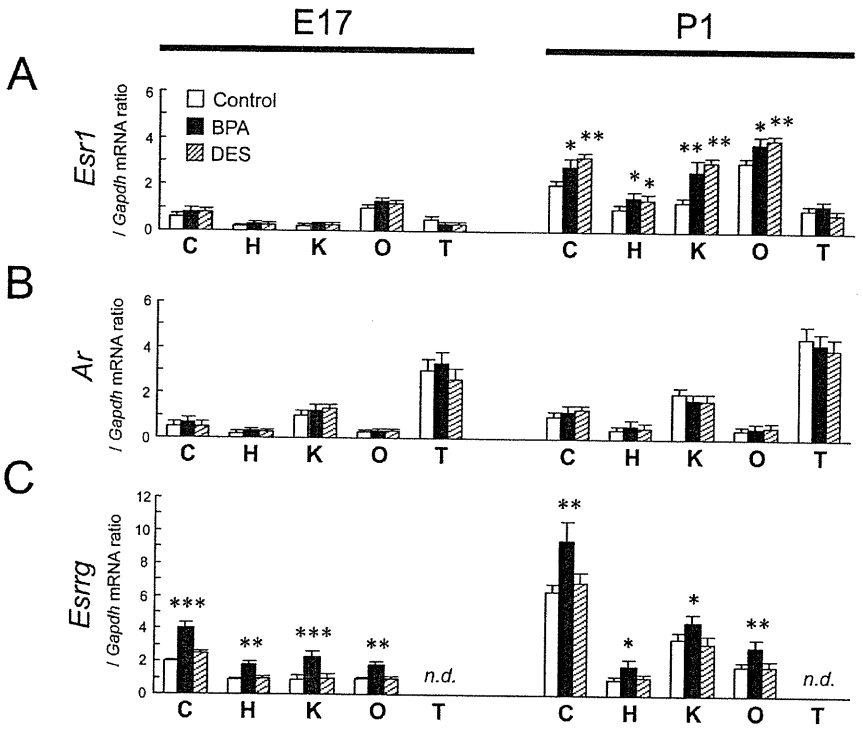

FIG. 7. BPA-specific up-regulation of *Esrrg* mRNA in sex steroid hormone-related organs. The relative mRNA expressions of *Esr1* (**A**), *Ar* (**B**), and *Esrrg* (**C**) were determined in sex steroid hormone-related organs of the untreated control (open bar), BPA-treated UGS (closed bar), and DES-treated UGS (slashed bar) at E17 and P1. C, cerebellum; H, heart; K, kidney; O, ovary; T, testis; *n.d.*, not detected. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control.

tissue recombination model, the female VMP induces prostate development in response to androgens [32], suggesting that cells within the female VMP have prostatic-inductive activity. Moreover, an earlier tissue recombination study showed that the ability of the female UGS to respond to androgens in forming prostate was gradually lost between P1 and P5 [33]. These results suggest strongly that androgen-responsive regulatory molecules are expressed constitutively even in the female VMP. Although the female VMP forms in the absence of androgens, androgen receptor (AR) expression was observed in the neonatal female VMP in a pattern similar to that observed in the male VPM [34]. Therefore, the BPA-specific increase in E_2 levels might interact with the intracellular AR signaling in both the male VPM and the female VMP. However, to our knowledge,

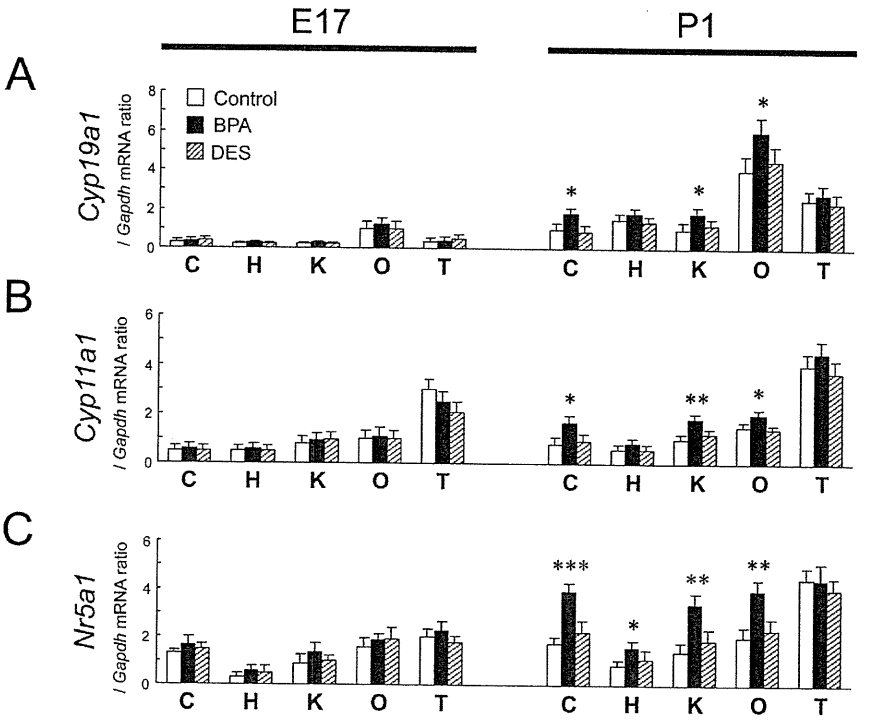

FIG. 8. BPA-specific up-regulation of steroidogenic enzyme and sex-determining gene mRNA in sex steroid hormone-related organs. The relative mRNA expressions of *Cyp19a1* (**A**), *Cyp11a1* (**B**), and *Nr5a1* (**C**) were determined in sex steroid hormone-related organs of the untreated control (open bar), BPA-treated UGS (closed bar), and DES-treated UGS (slashed bar) at E17 and P1. C, cerebellum; H, heart; K, kidney; O, ovary; T, testis. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control.