

Kyoto University graduate school of medicine: tradition and modernity harmonized

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Keywords History · Kyoto · Medical school

Beginning of the medical school

The city of Kyoto boasts a proud heritage tracing back to 794 AD which marked the beginning of its reign as the capital of Japan. It remained the capital for more than a thousand years until 1868, when the Meiji emperor proclaimed the “Meiji restoration” and all executive government functions, along with the title of capital, were transferred to *Edo* (known today as Tokyo). In 1897, roughly three decades after this transition, Kyoto University was formally established as the second Imperial University, followed two years later by the erection of the national medical school. In its 110-year history, Kyoto University has, among other things, made invaluable contributions to the promotion and advancement of the basic sciences. Since its establishment, six scientists from Kyoto University (graduate or faculty member) have been awarded the Nobel Prize in the fields of physics, chemistry and medicine (Hideki Yukawa 1949, Shin-ichiro Tomonaga 1965, Ken-ichi Fukui 1981, Susumu Tonegawa 1987, Ryoji Noyori 2001 and Toshihide Masukawa 2008).

These monumental achievements aside, the Medical School at Kyoto University has a very modest and humble beginning (Fig. 1). It only started with the fundamental departments of anatomy, physiology, medical chemistry,

pathology, pharmacology, hygiene, legal medicine, internal medicine, surgery, psychiatry, obstetrics and gynecology, pediatrics, ophthalmology, and dermatology. In 1919, the total number of departments was expanded to 24, in accordance with an amended national policy. Among the countless outstanding research carried out prior to World War II, the extensive research carried out by Dr. Hiroshi Fujinami, a professor of pathology, is revered even today for its impact on modern medicine. Prof. Fujinami not only discovered *Schistosoma japonicum*, the causative parasite of an endemic disease (Katayama disease), but also found that myxosarcoma in chickens can be transmitted by a filterable agent from the lesion. These findings paved the way towards development of the current concept of viral oncogenesis.

Medical school after World War II

In 1947, after WWII, the Japanese educational system underwent massive reform and with that the Kyoto Imperial University was formally renamed Kyoto University. The establishment of the Graduate School of Medicine followed soon after in 1955, and covered five major subject areas of medicine: physiology, pathology, social medicine, internal medicine, and surgery. Since its inception, each and every single member of the faculty was charged with the responsibility of training and educating both graduate and undergraduate students. While many distinguished faculty members are widely recognized for their pioneering research, some warrant exceptional recognition. Prof. Kozo Okamoto is a monumental example. During his tenure at the depart-

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Fig. 1 Histology laboratory in the Medical School at Kyoto University, ca, 1914



ment of pathology, he established a strain of spontaneously hypertensive rats (SHR) which are actively used in studies of metabolic syndrome today. From the department of medical chemistry, a number of noteworthy biochemists made groundbreaking discoveries in the emerging field of biochemistry and were recognized at an international level for their resounding success. Prof. Osamu Hayaishi discovered “oxygenase” and assumed a central role in the development of molecular enzymology. Prof. Shosaku Numa cloned a series of opioid peptide genes and erected the novel field of molecular neurobiology. Countless biochemists that would eventually become the symbols of progress in their respective fields were trained under the tutelage of these two monumental figures in biochemistry. Among the many are Professor Yasutomi Nishizuka, who discovered protein kinase C; Prof. Tasuku Honjo who discovered AID, an essential molecule involved in class-switch recombination; and Prof. Shigetada Nakanishi who successfully cloned the metabolic glutamate receptor (mGluR) which launched a new era of molecular neuroscience. Prof. Shu Narumiya, the former dean, has also made a lasting mark in modern medical biochemistry. Finally, Prof. Shoichiro Tsukita who passed away at a young age provided significant contributions to the greater understanding of the structural basis of cellular tight junctions by discovering claudin and occludin. All of these professors have been awarded the Japan Academy Award—the highest and most prestigious academic honor which can be received in Japan. In this manner, the basic research departments at the Graduate School of Medicine at Kyoto University have always had

a strong influence on the advancement of the medical sciences in both the foreign and domestic arena.

University Hospital

For a long period following the foundation of the University Hospital, each clinical department was granted its own building consisting of an outpatient clinic, inpatient ward, physician’s office and a laboratory.

Under this previous system, the university hospital was not a consolidated healthcare facility placed under the comfort of one roof, but a compound lined with multiple wooden buildings—each catering to a specific branch of medicine. This style of healthcare prevailed at Kyoto University until 1958 when the construction of an avant-garde centralized hospital building began. This modern university hospital with about 1,300 beds is now composed of 31 clinical divisions, 21 central clinical centers, a pharmacy, and affiliated research centers that would conduct front-line research in aspects such as translational research, clinical trial and management, and medical informatics (Figs. 2 and 3).

Many recent advances in the field of clinical medicine have been lead by our faculty and staff. Prof. Kiyoshi Takatsuki identified a novel clinical entity of adult T-cell leukemia (ATL); Prof. Yorio Hinuma and his colleague discovered the causative agent of ATL, a human retrovirus HTLV-1. Prof. Kazuo Honjou successfully carried out the world’s first whole resection of a pancreas. In addition to this, Prof. Koichi Tanaka successfully negotiated one of the first liver transplantation procedures from a live donor in

Fig. 2 Aerial view of the medical campus, 2005



Japan. This is a vital feat considering the lack of organ availability from brain-dead donors in our country. Since then, Kyoto University hospital has assumed an active role in transplantation surgery and has since come to be known as one of the world's finest institutions in this field.

The present situation: Education in Medical School

The undergraduate school provides an intensive 6-year curriculum in medicine. Our students undergo one of the most rigorous selection procedures in the world and only 100 of the most highly competitive candidates are accepted. For

the first 18 months, students are required to achieve at least 54 credits in the liberal arts (including foreign languages). Next, in the latter half of their second year, students are introduced to anatomy and histology. The third-year curriculum requires three introductory courses (medical biology, radiation biology and medical informatics literacy), ten lectures in core subjects (histology, gross human anatomy, embryology, molecular cell biology, physiology, neuroscience, etc.) and nine lectures in advanced subjects (pathology, microbiology, immunology, pharmacology, social medicine, etc.). These courses are mandatory and students who fail to obtain passing marks in these subjects may not advance to the next level. Those who move on to their fourth year curricula are exposed to



Fig. 3 University Hospital, 2008

coursework in 27 clinical related contexts which include cardiovascular medicine, hematology, respiratory diseases and gastroenterology.

Traditional classroom instruction has its limits, and in order to promote the acquisition of sound medical knowledge, the first semester for fourth year students are cleared of any mandatory coursework. Instead, students are encouraged to seek brief apprenticeships at active laboratories within Kyoto University, or at any other domestic or foreign academic institutions. This free-semester system provides the ideal setting for each student to gain first-hand exposure in medical science and research. Students who take an active interest in basic research via this system are given the option of switching tracks midstream to an MD/PhD course. Those interested in entering the MD/PhD course take an entrance examination at the end of their fourth years and, should they pass, enter the Graduate School of Medicine to conduct PhD research for a period of 4 years. After completion of the PhD course, the students return to their fifth year of medical school. Upon completion of the medical school curriculum, they are granted an MD. In 2009, two students are in the MD/PhD program in basic pathology and cell biology.

For students who continue with the standard—and not MD/PhD—course of medical school, the end of the fourth year of medical school marks a defining moment. They must successfully negotiate two major assessments: the CBT and OSCE. The CBT, or computer-based test, is designed for the sole purpose of measuring basic knowledge in medicine. Conversely, the OSCE (objective structured clinical examination) is implemented to gauge the students' aptitude in fundamental clinical skills. Students who pass the CBT and OSCE proceed to their fifth year of medical school. Here, students with demonstrated clinical skills and medical knowledge are divided into small groups. In these groups, the students will perform clinical rounds in the hospital under the guidance of a seasoned staff physician. Most of the major clinical subjects are mandatory; however, elective courses are also offered. The emphasis now is placed on fostering physician scientists and clinical doctors with a wide range of knowledge and interests. The knowledge and skills honed during the 6-year curriculum are put to a final test in what is known as the graduation examination. This is different from the licensing examination and students who pass this examination formally graduate from medical school.

The present: Education in the Graduate School of Medicine

The scope and frame of the graduate school far surpasses that of the medical school. Kyoto University has 13

affiliated research institutes and some of these are deeply involved in medical science. Some of our more recent accolades include the world's first establishment of iPS cells by Prof. Shinya Yamanaka at the Institute of Frontier Medical Sciences (IFMS), and the discovery of HTLV-1 at the Institute for Virus Research (IVR). The Graduate School of Medicine now consists of over 150 independent laboratories headed by full professors including most of the divisions at IFMS, IVR and some laboratories from the institute for Chemical Research and the Research Reactor institute. In the year 2000, the School of Public Health was founded and has since been integrated into the Graduate School of Medicine. Similarly, the School of Health Sciences has also joined the ranks of the graduate school.

The PhD course at the Kyoto University Graduate School of Medicine has a diverse background with regard its proportion of international students and researchers. The student population for the graduate school is 700, of which more than 100 are accounted for by foreign students from countries such as China, Thailand, Bangladesh, Korea, Myanmar, Vietnam, USA, Germany, France, and Nigeria. Graduate students are assigned to and carry out PhD research at any one of the more than 150 laboratories actively conducting research at the Graduate School. While the students are required to obtain a minimum amount of credits in required coursework, they are mainly assessed by the publication of original articles in international academic journals of high standing and recognition. Numerous papers have been published by our promising graduate students in leading journals such as *Nature*, *Cell*, *Science* and *PNAS*.

More recently, the "career path unit" was established where outstanding postdoctoral fellows or young faculty members can be promoted to the position of Associate Professor. Those who are selected by a committee are granted a research stipend, independent laboratory space and a couple of laboratory technicians as principal investigator (PI). In the last 5 years, five such PIs have been promoted to the status of full professors in Japan.

To facilitate inter-specialty collaboration among individual laboratories, we have initiated several projects supported by generous sums provided by national funding. The COE (Center of Excellence) project is a prime example and promotes, among many other things, collaboration in the field of animal disease models and regenerative medicine (tissue engineering). Collaborative studies on lifestyle disease and cancer by the recently established School of Public Health, Translational Research Center, and Human Genome Research Center are now also underway and are expected to lead pave a path for the advancement of the clinical science not only in Japan but on the international stage as well.

Conclusion

Kyoto University Medical School, one of Japan's oldest and most revered institutions, has produced a great number of leading physician-scientists and basic medical scientists. Training the next generation of leaders in the medical sciences will always be our responsibility, but another noteworthy

endeavor that is implied in our global standing is the cultivation and establishment of a new era of medical science and clinical medicine; hence the distinction in the manner of reference to our institution. We refer to our institution as the Graduate School of Medicine and not the School of Medicine due to our inherent duty to perform advanced medical research for the betterment of mankind.

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Prostanoids and inflammation: a new concept arising from receptor knockout mice

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Abstract Prostanoids including various types of prostaglandins and thromboxanes are arachidonate metabolites produced and released in response to a variety of physiological and pathological stimuli and function to maintain the body homeostasis. Since cyclooxygenase, the enzyme initiating their biosynthesis, is inhibited by aspirin-like antipyretic, anti-inflammatory, and analgesic drugs, contribution of prostanoids to acute inflammation such as fever generation, pain sensitization, and inflammatory swelling has been recognized very early. On the other hand, since aspirin-like drugs generally show little effects on allergy and immunity, it has been believed that prostanoids play little roles in these processes. Prostanoids act on a family of G-protein-coupled receptors designated PGD receptor, PGE receptor subtypes EP1–EP4, PGF receptor, PGI receptor, and TX receptor to elicit their actions. Studies using mice deficient in each of these receptors have revealed that prostanoids indeed function in the above aspirin-sensitive processes. However, these studies have also revealed that prostanoids exert both pro-inflammatory and anti-inflammatory actions not only by acting as mediators of acute inflammation but also by regulating gene expression in mesenchymal and epithelial cells at inflammatory site. Such dual actions of prostanoids are frequently seen in immune and allergic reactions, where different type of prostanoids and their receptors often exert opposite actions in a single process. Thus, a new concept on the role of prostanoids in inflammation has arisen from studies using the receptor knockout mice.

Keywords Prostaglandin · Prostaglandin receptor · Inflammation · Immunity · Allergy

Introduction

Prostanoids including prostaglandin (PG) D₂, prostaglandin E₂ (PGE₂), prostaglandin F₂α (PGF₂α), prostacyclin (PGI₂), and thromboxane (TX) A₂ are produced from arachidonic acid by the sequential actions of cyclooxygenase (COX) and respective synthases. They are formed and released in response to various, often noxious, stimuli, function in a paracrine and autocrine fashion in the vicinity of their production, and serve to maintain local homeostasis in the body. They act on their cognate receptors on the surface of target cells to exert their actions. There are eight types and subtypes of receptor for prostanoids designated PGD receptor (DP), EP1, EP2, EP3, and EP4 subtype of PGE receptor, PGF receptor (FP), PGI receptor (IP), and TX receptor (TP) [1]. All of them are G-protein-coupled receptors (GPCRs) and constitute a prostanoid receptor family in the superfamily of GPCRs. In addition, there is another GPCR termed chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) that responds to PGD₂ but belongs to the family of chemokine receptors. Main signal transduction of each of these receptors is a rise in intracellular cyclic adenosine monophosphate (cAMP) concentration via G_s in DP, EP2, EP4, and IP, a rise in intracellular free calcium ion concentration via G_q or other G protein in EP1, FP, and TP and a decrease in intracellular cAMP concentration via G_i in EP3 and CRTH2, though most of them couple to more than one G protein and more than one signaling pathway. Mice deficient in each of these receptors individually were generated and subjected to various

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analyses. Furthermore, these receptors have been used as a panel in screening of chemical library, and agonists and antagonists highly selective to each member have been developed. Analyses using these knockout mice and applying selective compounds to wild-type mice have revealed roles that each receptor plays in various physiological and pathophysiological processes. Here, I focus mainly on findings my own group have obtained through such studies (Table 1) and discuss new concepts of prostanoids in inflammatory processes arising from these findings. The findings on the knockout mice reviewed were those on the mice that were backcrossed for more than five generations onto the C57BL/6 background except for EP4-deficient mice, which were in the mixed genetic background of 129SV and C57BL/6, or unless specified in the text. For other aspects of the prostanoid receptor actions, please refer to a more comprehensive review [1].

Prostanoid receptors as mediators of classic signs of acute inflammation

Local reddening, heat generation, and swelling are classic signs of acute inflammation, which are caused by increased blood flow and vascular permeability. Inhibitory effects of nonsteroidal anti-inflammatory drugs (NSAIDs) on these signs and vasodilatory action of prostanoids such as PGI₂ and PGE₂ implicated prostanoids as mediators of the vascular responses in acute inflammation. Indeed, using knockout mice deficient in each of prostanoid receptors

Table 1 Summary of actions of prostanoids and their receptors in inflammatory processes discussed in this review

PGD ₂ /DP:	facilitates allergic inflammation [18], suppresses Langerhans cell mobilization and migration [46]
PGD ₂ /CRTH2:	facilitates allergic inflammation [28], negatively regulate IL-5 production [29]
PGE ₂ /EP1:	facilitates Th1 differentiation [48], peripheral hyperalgesia [8]
PGE ₂ /EP2:	mediates pleural exudation [3] and central hyperalgesia [10], facilitates progression of arthritis [12], facilitates Th1 differentiation and Th17 expansion [37]
PGE ₂ /EP3:	mediates pleural exudation [3], fever generation [5], and peripheral hyperalgesia [7], negatively regulates allergic inflammation [22–24]
PGE ₂ /EP4:	mediates peripheral hyperalgesia [9], facilitates progression of arthritis [12], suppresses intestinal inflammation [13], facilitates Th1 differentiation and Th17 expansion [37], promotes Langerhans cell mobilization and migration [45]
PGI ₂ /IP:	mediates inflammatory swelling [2], peripheral hyperalgesia [2, 8], and pleural exudation [3, 4], facilitates progression of arthritis [12], attenuates IgE production [30]
TXA ₂ /TP:	negatively regulates interaction of dendritic cells and T cells [47]

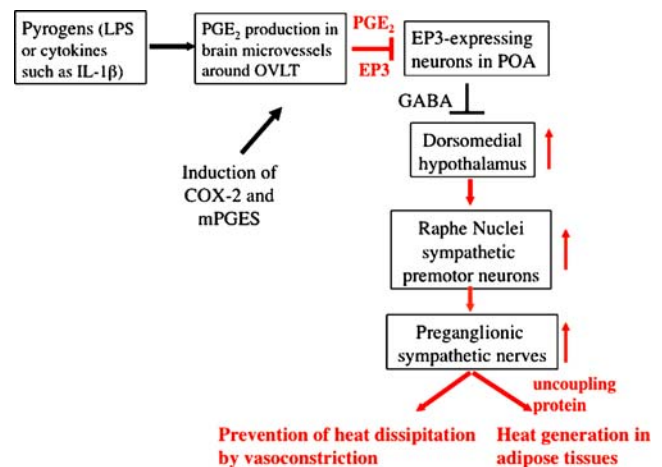


Fig. 1 A current model for the neural pathway of fever generation. When PGE₂ is formed in brain microvessels in the organum vasculosum laminae terminalis (OVLTL), it enters the brain and acts on the EP3-expressing neurons in the preoptic area (POA). The EP3 stimulation inhibits the GABAergic inhibitory transmission of these POA neurons and liberates the downstream neural pathway, ultimately leading to sympathetic stimulation in peripheral tissues (shown in red)

individually, Murata et al. found the ~50% reduction in carrageenin-induced paw swelling in IP-deficient mice, a reduction similar in magnitude to that achieved by treatment with NSAIDs in wild-type mice [2]. Yuhki et al. showed that EP2 and EP3 as well as IP mediate pleural exudation in carrageenin-induced mouse pleurisy at 1–5 h after the carrageenin injection [3]. Yuhki et al. further showed that the PGI₂–IP pathway is the main prostanoid signaling for exudate formation in zymosan-induced pleurisy [4]. These results are consistent with the role of prostanoids as mediators of acute inflammation and suggest that PGE₂ and PGI₂ elicit inflammatory responses in a context-dependent manner, that is, dependent on the type of stimulus and the site of the body. As for other signs of acute inflammation, EP3 was identified as the receptor mediating febrile response [5], and the neural pathway from pyrogen stimulation to fever generation has been elucidated in detail (Fig. 1; [6] and references therein). In pain sensation, various types of prostanoid receptors, IP, EP1, EP3, and EP4, are involved in peripheral hyperalgesia in a context-dependent manner [2, 7–9], one mechanism being lowering the threshold of TRPV1 cation channel by EP1 and IP [8]. In addition, EP2 inhibits glycinergic inhibitory neurotransmission in the spinal cord and causes central hyperalgesia [10, 11].

Gene expression-dependent pro-inflammatory actions of prostanoids

The above studies thus substantiated the role of prostanoids in acute inflammation. However, one novel and important

message obtained by knockout mouse studies is that prostanoids exert both pro-inflammatory and anti-inflammatory actions through regulation of gene expression in relevant tissues. Honda et al. subjected mice deficient in prostanoid receptors in the DBA/1J background to collagen-induced arthritis, an animal model of rheumatoid arthritis [12]. Whereas the incidence was unaffected, the extent and progression of arthritis were markedly suppressed in IP-deficient mice as well as in EP2-deficient mice treated with an EP4 antagonist, indicating that PGI₂–IP signaling and PGE₂ signaling through EP2 and EP4 mediate joint inflammation in this model. Further analysis revealed that both PGI₂ and PGE₂ pathways function in conjunction with interleukin (IL)-1 β and enhance expression of arthritis-related genes, including those for IL-6, vascular endothelial growth factor-A, and receptor activator of NF-kappa B ligand, in synovial fibroblasts and thereby contribute to arthritic inflammation, bone destruction, and pannus formation (Fig. 2). Collagen-induced arthritis thus represents an example in which prostanoids elicit pro-inflammatory actions through expression of pro-inflammatory genes.

Gene expression-dependent anti-inflammatory actions of prostanoids

An example of the prostanoid action contrary to the above was obtained by analysis of the knockout mice subjected to dextran sodium sulfate (DSS)-induced colitis, an animal model of ulcerative colitis [13]. Ulcerative colitis is one of inflammatory bowel diseases (IBD) and is characterized by inflammation in the large intestine associated with diarrhea,

occult blood, abdominal pain, weight loss, anemia, and leukocytosis. Studies in humans have implicated impaired mucosal barrier function, pronounced innate immunity, production of pro-inflammatory cytokines, and the activation of CD4⁺ T cells in the pathogenesis. Administration of NSAIDs often triggers or worsens the colitis [14], which was confirmed experimentally by studies on COX-deficient mice subjected to DSS-induced colitis [15], indicating that COX-derived prostanoids contribute to the defense against intestinal inflammation. Based on these findings, Kabashima et al. examined the susceptibility of mice deficient in each of prostanoid receptors to DSS treatment [13]. They found that only EP4-deficient mice developed severe colitis in response to treatment with 3% DSS. They then reproduced this phenotype in wild-type mice by administration of an EP4-selective antagonist and confirmed this finding pharmacologically. EP4 deficiency was shown to result in impairment of mucosal barrier function that was associated with epithelial loss, crypt damage, and accumulation of neutrophils and CD4⁺ T cells in the colon. DNA microarray analysis revealed increased expression of genes associated with immune responses and reduced expression of genes associated with mucosal repair and remodeling in the colon of EP4-deficient mice. Thus, the PGE₂–EP4 signaling appears to maintain intestinal homeostasis by preserving mucosal integrity and downregulating immune responses through regulation of gene expression. It is intriguing that the same receptor, EP4, exerts an anti-inflammatory action in intestinal inflammation and a pro-inflammatory action in arthritis, emphasizing the context-dependent roles of prostanoid signaling.

Prostanoid receptors in allergic inflammation

Pro-inflammatory and anti-inflammatory actions of prostanoids sometimes operate in a single disease process. One example is allergic inflammation, where the PGD₂–DP signaling and the PGE₂–EP3 signaling exert antagonistic actions. The type I allergic reaction underlies the pathogenesis of bronchial asthma, atopic dermatitis, and anaphylactic shock. Affected individuals produce immunoglobulin (Ig) E antibodies to allergens such as those derived from house dust mites and plant pollen. Exposure to those allergens results in cross-linking of IgE receptors on the surface of mast cells, the consequent activation of these cells, and the development of an allergic reaction. The Th2 subset of T lymphocytes and their cytokines are important mediators of IgE production as well as the development of allergic disease. Various prostanoids are produced during the initial activation of mast cells and subsequent disease development. PGD₂ is a major prostanoid produced by activated mast cells [16] and is released in large amounts

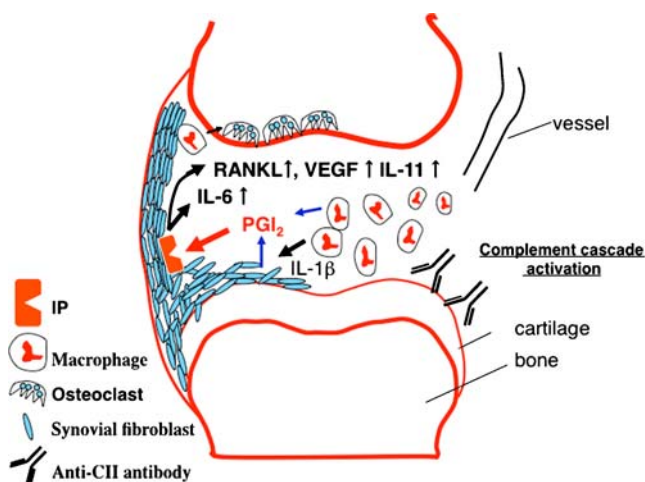


Fig. 2 A model of prostanoid actions in the inflammatory cascade of collagen-induced arthritis. In the arthritic joint, PGI₂ acts on IP in synovial fibroblasts and facilitates the IL-1 β -induced expression of genes such as IL-6, RANKL, VEGF, and IL-11. PGE₂ functions similarly by acting on EP2 and EP4

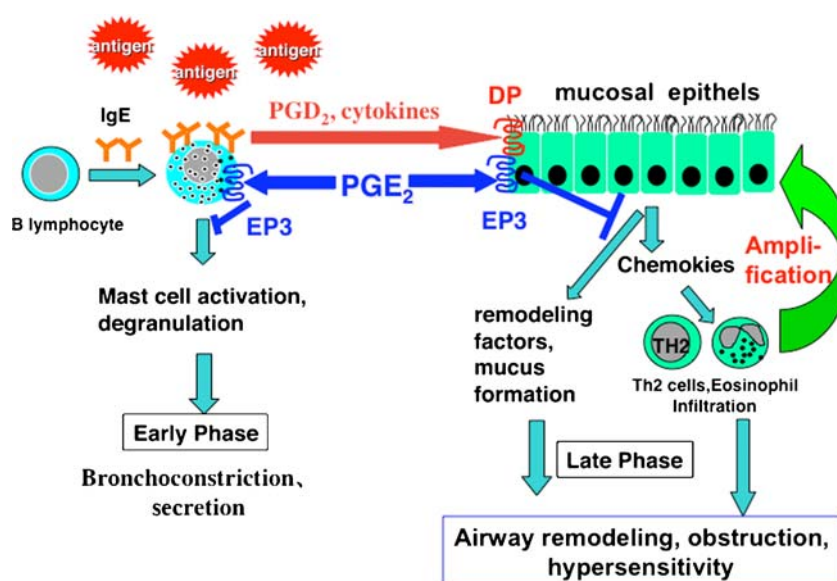
during asthmatic attacks in certain patients [17]. The role of PGD_2 in allergic asthma long remained unclear, however. Matsuoka et al. examined this issue by applying the ovalbumin (OVA)-induced asthma model to DP-deficient mice [18]. Sensitization and aerosol challenge of $\text{DP}^{-/-}$ mice with OVA induced increases in the serum concentration of IgE similar to those observed in wild-type mice. However, the DP-deficient animals developed substantially reduced asthmatic responses in this model; the concentrations of Th2 cytokines and the extents of lymphocyte accumulation and eosinophil infiltration in the lungs after OVA challenge were greatly reduced in the mutant animals compared with those apparent in the wild type. These observations thus indicate that PGD_2 is a mast cell-derived mediator that serves to mediate asthmatic responses. This conclusion is supported by single nucleotide polymorphism (SNP) analysis of the DP gene (*PTGDR*) in humans. This gene is located at chromosome 14q22.1, a region that has been associated with asthma and atopy. Oguma et al. [19] examined SNPs of *PTGDR* in Caucasian and black individuals with asthma and control subjects. They identified three haplotypes consisting of four SNPs in the promoter region of the gene. They further found that these haplotypes show a different promoter activity and that the promoter activity of these haplotypes is significantly correlated with susceptibility to asthma.

The above observations suggest that PGD_2 signaling facilitates allergic responses not only in mice but also in humans. However, if PGD_2 is the only prostanoid that functions in allergic asthma, administration of NSAIDs would be expected to ameliorate asthmatic symptoms. Instead, NSAIDs are either without effect or induce severe attacks in certain asthmatic patients, a syndrome known as aspirin-induced asthma [20]. This discrepancy might be explained by the existence of a prostanoid other than PGD_2 that negatively modulates allergic reactions. The most likely candidate for such a prostanoid is PGE_2 , given that PGE_2 has been known for some time to exert anti-allergy effects in some contexts [21]. Kunikata et al. subjected mice deficient in each EP subtype individually to the OVA-induced asthma model and examined their responses [22]. Among the four knockout mouse strains, only EP3-deficient mice exhibited substantially exaggerated airway inflammation compared with that observed in their wild-type counterparts while showing similar plasma concentrations of anti-OVA IgE. The EP3-deficient animals also manifested an enhanced passive cutaneous anaphylaxis reaction. These results thus implicated PGE_2 -EP3 signaling in suppression of mast cell activation. Consistent with this conclusion, an EP3-selective agonist was found to inhibit the antigen-induced release of histamine and leukotrienes from sensitized lung tissue in vitro and to suppress airway inflammation in OVA-challenged mice in vivo. Previously,

aspirin-induced asthmatic attack was explained by diversion of arachidonic acid metabolism from the COX pathway to the lipoxygenase pathway [20]. Our findings indicate that this is not simple diversion of the substrate from one pathway to the other, but due to enhancement of the lipoxygenase pathway by aspirin-mediated suppression of the PGE_2 -mediated mast cell inactivation. Thus, the PGE_2 -EP3 pathway acts through mast cell inactivation. However, the mast cell inactivation is not the only mechanism of the EP3-mediated inhibition of allergic inflammation. The EP3 agonist was most effective when administered subcutaneously 3 h after OVA challenge, indicating that the major site of EP3 action is at a step (or steps) downstream of mast cell activation. Further analysis revealed that administration of the EP3 agonist suppressed induction of the expression of various asthma-related genes, including those for chemokines and tissue remodeling factors, in the lung, and that EP3 is co-expressed with some of these molecules in the airway epithelium. On the basis of these findings, Kunikata et al. suggested that PGE_2 produced during allergy acts at EP3 on both mast cells and airway epithelial cells, thereby blunting activation of mast cells and impeding progression of the allergic reaction by inducing downregulation of the expression of relevant genes in the airway epithelium. DP is also expressed in the airway epithelium, and given its opposing mechanism of signal transduction relative to that of EP3, it possibly facilitates the asthmatic reaction by increasing expression of these genes. Recently, suppression of allergic response by EP3 has also been found in mouse models of allergic conjunctivitis and contact hypersensitivity of the skin [23, 24], where expression of EP3 is found in epithelial cells of the conjunctiva and keratinocytes of the skin, respectively. The roles of the PGD_2 -DP and the PGE_2 -EP3 pathways in allergic reactions suggested by these studies are depicted in Fig. 3.

In addition to the above actions of DP and EP3, there are other actions in allergy in which prostanoids and their receptors are involved. For example, PGD_2 may also function in allergy by acting at the receptor CRTH2. CRTH2 can bind PGD_2 and is expressed in cells important in allergy such as Th2 lymphocytes, eosinophils, and basophils [25]. Given that stimulation of CRTH2 induces chemotaxis of these cells in vitro, it has been suggested that CRTH2 facilitates allergic inflammation. Indeed, administration of selective agonists for CRTH2 to the airway or painting of these substances on the skin of sensitized animals was found to augment infiltration of inflammatory cells into the lungs and skin, respectively [26, 27]. The generation of CRTH2 knockout mice allowed further examination of the importance of the PGD_2 -CRTH2 pathway in the natural course of allergy. Satoh et al. [28] used CRTH2-deficient mice that were backcrossed more

Fig. 3 Antagonism of the PGD_2 -DP signaling and the PGE_2 -EP3 signaling in allergic inflammation. In the ovalbumin-induced bronchial asthma model, the PGD_2 -DP pathway, together with various cytokines stimulates and the PGE_2 -EP3 pathway, suppresses the late phase of allergic inflammation through up- and downregulation of expression of inflammation-related genes in the airway epithelial cells, respectively. The PGE_2 -EP3 pathway also acts on mast cells and suppresses their activation



than ten generations to the Balb/cJ background and found that allergic inflammation associated with IgE-induced dermatitis was suppressed in these mice. On the other hand, Chevalier et al. [29] subjected to the OVA-induced asthma model the CRTH2 knockout mice they generated in the mixed genetic background of 129SV and C57BL/6 and found that airway inflammation and eosinophil infiltration were augmented in the knockout mice. The latter study also showed that IL-5 production by activated T cells from CRTH2-deficient mice in vitro was increased compared with that observed with wild-type cells. These results indicated that CRTH2 indeed functions to facilitate allergy in situ at the site of inflammation but that this receptor also regulates cytokine production in the early phase of allergy development, raising the question as to whether suppression of this pathway would result in an overall beneficial effect in patients. Application of the OVA-induced asthma model to mice deficient in other prostanoid receptors revealed that airway inflammation was also augmented in IP-deficient mice. In contrast to EP3-deficient mice, however, $\text{IP}^{-/-}$ mice exhibited substantially higher plasma concentrations of antigen-specific and total IgE, indicating that PGI_2 -IP signaling functions in sensitization to IgE production [30]. Thus, prostanoids and their receptors function at various steps of allergic inflammation.

Prostanoid receptors in immune inflammation

Inflammation can be caused by a variety of stimuli, one of which is immune stimulus. It is known that autoaggressive helper T (T_H) cells induce tissue damage and cause inflammation, and these actions are believed to play roles in pathogenesis of immune diseases such as multiple

sclerosis and rheumatoid arthritis [31–33]. Among three effector T_H subsets, $\text{T}_\text{H}17$ cells or both $\text{T}_\text{H}1$ and $\text{T}_\text{H}17$ cells mediate tissue damage, inflammation, and autoimmunity [31–33]. $\text{T}_\text{H}1$ and $\text{T}_\text{H}17$ cells are characterized by their expression of interferon- γ and IL-17, respectively. $\text{T}_\text{H}1$ differentiation is induced by IL-12 and $\text{T}_\text{H}17$ differentiation is induced by transforming growth factor- β and IL-6 and expanded by IL-23. Notably, IL-12 and IL-23 shares the common p40 subunit. Recent reports indicate that PGE_2 is involved in differentiation and expansion of these TH subsets and therefore in elicitation and progression of immune diseases. It is known from 1980s that PGE_2 suppresses $\text{T}_\text{H}1$ differentiation through a rise in cAMP, and the receptors mediating this action have been identified as EP2 and EP4 [34–36]. However, T cell suppression by PGE_2 generally requires high concentration of this PG and has been shown mostly, if not exclusively, in in vitro culture systems and is rarely seen in vivo in intact animals, raising a possibility that PGE_2 acts differently in in vivo immune responses. Yao et al. [37] reexamined this issue and found that, on the contrary to the previous findings, PGE_2 can induce $\text{T}_\text{H}1$ differentiation through EP2 and EP4 under the strengthened TCR stimulation, and this action is dependent on phosphatidylinositol (PI)-3 kinase activation and not cAMP. They also found that PGE_2 facilitates $\text{T}_\text{H}17$ expansion by IL-23 through the receptors, EP2 and EP4, but this action is mediated by cAMP and not PI-3 kinase. The same group further found that production of IL-23 by activated dendritic cells (DCs) requires PGE_2 and this action is also dependent on cAMP. It is now known that some GPCRs transduce signals not only through heterotrimeric G proteins but also through β -arrestin, which recruits c-Src and signals to PI-3 kinase, and EP4 is one of such GPCRs [38]. Thus, PGE_2 utilizes different signaling

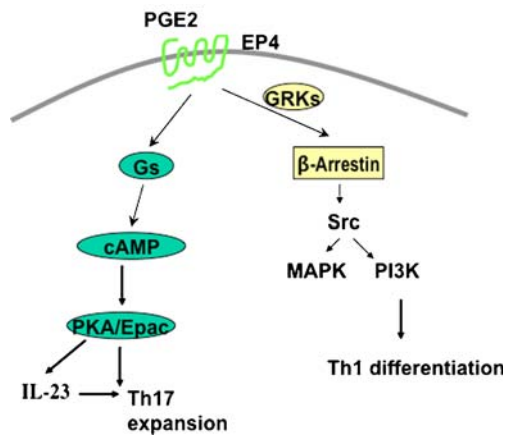


Fig. 4 EP4 signaling in Th1 differentiation and Th17 expansion. The PGE₂–EP4 signaling facilitates Th1 differentiation and Th17 expansion through utilizing the PI-3 kinase pathway in T cells and the cyclic AMP pathway in T cells and DCs, respectively. See the text for details

modules of EP2/4 and facilitates T_H1 differentiation and T_H17 expansion (Fig. 4). To verify that these actions of EP4 operate *in vivo* in immune disorders, Yao et al. [37] used mouse models of allergic skin disease and multiple sclerosis, that is, contact hypersensitivity (CHS) and experimental autoimmune encephalomyelitis. They subjected mice to these models and examined effects of an EP4 antagonist. The antagonist administration suppressed disease progression in both experiments, which was accompanied by reduced T_H1 and T_H17 cell accumulation in lymph nodes, confirming that the EP4-dependent step or steps are critical in differentiation or expansion of T_H1 and T_H17 cells and elicitation of diseases. Consistent with the findings by Yao et al., Ganea and collaborators activated bone marrow-derived CD11c⁺ DCs with lipopolysaccharide in the presence of exogenously added PGE₂ and found that

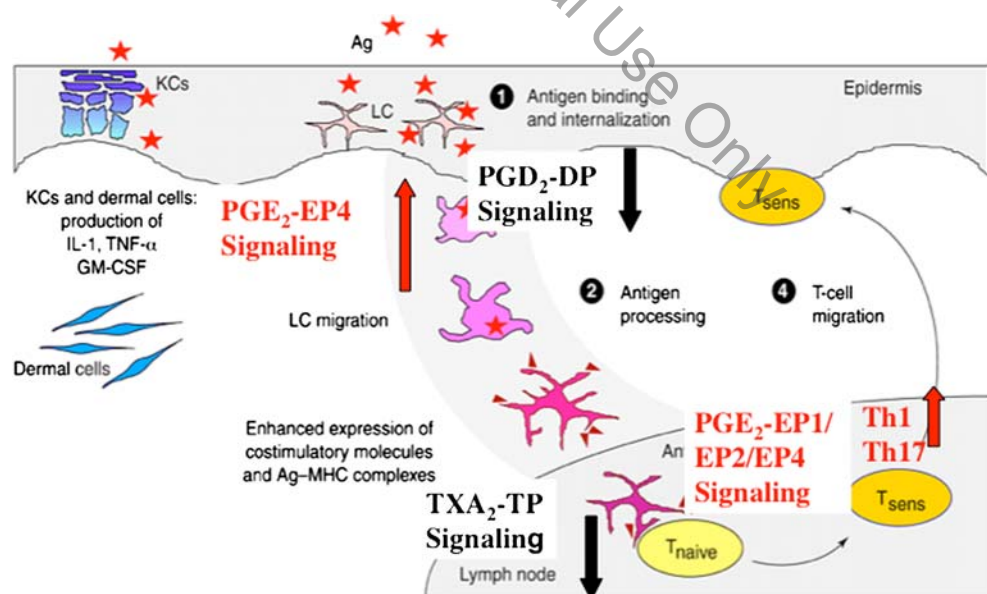
PGE₂ can augment IL-23 production by DCs by about two times [39, 40]. Stimulatory action of PGE₂ on T_H17 differentiation or expansion *in vitro* has been reported also in human peripheral blood mononuclear cells [41]. It is therefore likely that the PGE₂–EP4 signaling operates in immune diseases of humans. It is noted in this context that human EP4 was assigned as a susceptibility gene of Crohn’s disease [42, 43], in which involvement of IL-23 and T_H17 is indicated [44]. These findings appear contradictory with the EP4 actions to prevent DSS-induced colitis described above, because ulcerative colitis and Crohn’s disease are both IBD. However, intestinal inflammation in the DSS model is initiated by damage of intestinal epithelial cells, and EP4 functions to enhance growth of the epithelial cells to augment their barrier function

In addition to these actions in immune inflammation, the receptor knockout studies have revealed that prostanoids and their receptors work at various steps of immunization, exert actions often opposing each other, and regulate immune response and that general inability of NSAIDs to affect immune response can be due to suppression of these actions altogether. For example, EP4 facilitates [45] and DP suppresses [46] DC mobilization and migration, TP regulates interaction of DCs and naïve T cells to regulate the extent of immune response [47], and EP1 regulates the balance of T_H1 and T_H2 [48] (Fig. 5).

Concluding remarks

Studies using mice deficient in each of the prostanoid receptors have not only substantiated the role of prostanoids and their mechanisms in acute inflammation including fever generation and pain sensation but also have

Fig. 5 Prostanoid signaling in the sensitization phase of immune response. Actions of various prostanoid signaling pathways in immune response have been examined using the contact hypersensitivity (CHS) model or other form of skin immune response [37, 44–46], and their presumed sites of action are depicted in the hypothetical scheme of the sensitization phase of CHS illustrated by Grabbe and Schwarz [49]. See the text for details



revealed that prostanoids exert both pro-inflammatory and anti-inflammatory actions through regulation of gene expression in relevant tissues and that such actions are often seen in allergic and immune inflammations. The fact that prostanoids and their receptors exert these actions in a context-dependent manner and regulate various steps of inflammation indicates that context-dependent selective manipulation of each receptor signaling may beneficially control inflammatory responses of certain diseases better than current anti-inflammatory drugs.

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Translational research of novel hormones: lessons from animal models and rare human diseases for common human diseases

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Abstract Since the 1980s, a number of bioactive molecules, now known as cardiovascular hormones, have been isolated from the heart and blood vessels, particularly from the subset of vascular endothelial cells. The natriuretic peptide family is the prototype of the cardiovascular hormones. Over the following decade, a variety of hormones and cytokines, now known as adipokines or adipocytokines, have also been isolated from adipose tissue. Leptin is the only adipokine demonstrated to cause an obese phenotype in both animals and humans upon deletion. Thus, the past two decades have seen the identification of two important classes of bioactive molecules secreted by newly recognized endocrine cells, both of which differentiate from mesenchymal stem cells. To assess the physiological and clinical implications of these novel hormones, we have investigated their functions using animal models. We have also developed and analyzed mice overexpressing transgenic forms of these proteins and knockout mice deficient in these and related genes. Here, we demonstrate the current state of the translational

research of these novel hormones, the natriuretic peptide family and leptin, and discuss how lessons learned from excellent animal models and rare human diseases can provide a better understanding of common human diseases.

Keywords Natriuretic peptide family (ANP, BNP, CNP) · Leptin · Translational research · Animal models · Genetically engineered mice

Although a multitude of animal models have been developed to emulate various diseases, there are a few excellent animal models that mimic human disease remarkably well, such as spontaneously hypertensive rats (SHR) [1] and hereditary obese mice, ob/ob mice [2]. These models are very useful for translational research into the common human diseases, hypertension and obesity. Lessons from research on SHR, an excellent animal model for hypertension research, developed at Kyoto University led us to investigate the clinical importance of cardiovascular hormones and adipokines using appropriate animal models that mimic human diseases beyond species differences. In this review, we discuss the current state of translational research of the natriuretic peptide family and leptin and discuss the ways in which animal models and rare human diseases can educate about common human diseases.

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Translational research of natriuretic peptide family

The natriuretic peptide family consists of three structurally related peptides, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) [3]. The biological actions of natriuretic peptides are mediated by activation of two subtypes of membranous guanylyl cyclase (GC), GC-A and GC-B, leading to

intracellular accumulation of cyclic guanine monophosphate (cGMP) [4]. The rank order of potency to induce cGMP production via GC-A is $ANP \geq BNP \gg CNP$, while that via GC-B is $CNP > ANP \geq BNP$ [5]. Thus, ANP and BNP serve as endogenous ligands for GC-A, while CNP is specific for GC-B. A third natriuretic peptide receptor with no intracellular GC domain, dubbed the clearance receptor (C-receptor), is thought to be engaged in the receptor-mediated degradation of natriuretic peptides [4]. The ANP, BNP/GC-A system plays a pivotal role in the regulation of cardiovascular homeostasis, as demonstrated by their augmentation in various pathophysiological states such as heart failure [6–10], myocardial infarction [11, 12], cardiac hypertrophy [13, 14], and hypertension [15–17]. ANP and BNP are cardiac hormones secreted primarily by the atrium and ventricle of the heart, respectively [10, 17], with strong diuretic, natriuretic, and vasodilatory activities [6, 7, 10]. ANP and BNP are used in the treatment of heart failure [18, 19] and serve as sensitive biochemical markers for heart failure and cardiac hypertrophy [8–10]. ANP infusion therapy has currently reached a greater than 30% share among drugs given for acute congestive heart failure in Japan.

CNP, the third member of natriuretic peptide family, was first purified from porcine brain [20]. While CNP is the primary natriuretic peptide in the human brain [21], it is also produced by vascular endothelial cells [22–24] and macrophages [25]. This hormone functions in the regulation of vascular endothelial function and arteriosclerosis via local effects, not by acting as a circulating hormone [26–28]. These observations indicate that CNP acts as an autocrine/paracrine regulator and as a neuropeptide [21].

The distribution of the natriuretic peptide system overlaps with the distribution of the renin–angiotensin system [21, 29–33], prompting us to examine the functional relationship of the natriuretic peptide system and the renin–angiotensin system. We demonstrated an antagonistic relationship between these two systems, both in their peripheral functions as well as their central actions [34–39]. Furthermore, the natriuretic peptide system has therapeutic implication in vascular regeneration in patients with arteriosclerosis obliterans [40].

Mice with genetic alterations in the ANP, BNP/GC-A system

Genetically engineered mice are useful tools to study the complex phenotypic effects of an altered gene in living animals. Overexpression or deficiency of each member of the natriuretic peptide family or its receptors has been generated through transgenic (Tg) or knockout (KO) technologies [41–45]. We generated Tg mice expressing BNP under the control of the serum amyloid P (SAP)

component promoter, which targets hormone expression to the liver [43]. BNP-Tg mice exhibited a 100-fold increase in plasma BNP concentrations with concomitant elevations in plasma cGMP concentrations. These mice displayed significantly lower blood pressures and smaller hearts than non-Tg littermates. These results indicate that BNP functions in the long-term cardiovascular regulation and may be useful as a long-term therapeutic agent. In addition, the proteinuria and renal dysfunction observed in anti-GBM nephritis [46], the nephrosclerosis induced by subtotal nephrectomy [47], and the manifestations of diabetic nephropathy [48] were ameliorated in BNP-Tg mice compared to those in wild-type mice, indicating a possible application for the natriuretic peptide family in the treatment of renal disorders.

We also generated mice bearing a targeted disruption of the BNP gene [44]. At baseline, BNP-KO mice did not show any signs of systemic hypertension or ventricular hypertrophy; however, these animals developed multifocal fibrotic lesions within the cardiac ventricle even in the absence of additional stresses; these lesions increased in size and number in response to ventricular pressure overload, demonstrating that BNP is an antifibrotic factor acting within the ventricle of the heart as an autocrine/paracrine regulator for ventricular remodeling [44]. In addition to these cardiovascular manifestations, BNP-Tg mice exhibited marked skeletal overgrowth via endochondral bone formation [49]. Nevertheless, BNP-KO mice did not possess any skeletal abnormalities [44]. The skeletal overgrowth seen in BNP-Tg mice that express elevated plasma concentrations of BNP was similar to that seen in cartilage-specific CNP-Tg mice [49]. As the BNP/GC-A system does not have an abnormal skeletal phenotype [41, 42, 45], we postulated that the markedly increased circulating levels of BNP (100-fold greater than wild-type mice) may cross-react with GC-B to stimulate endochondral bone growth, even though the affinity of BNP for GC-B is lower than that for GC-A. This interpretation is supported by the finding that the skeletal overgrowth observed in BNP-Tg mice was not abrogated by a genetic deficiency of GC-A in BNP-Tg mice [50].

ANP transgenic mice expressing elevated levels of circulating ANP under the control of mouse transthyretin promoter [41] exhibited decreased arterial blood pressure without the induction of diuresis or natriuresis. ANP-KO mice and GC-A-KO mice displayed salt-sensitive and salt-resistant hypertension, respectively [42, 45]. Studies using GC-A-KO mice implicated the involvement of GC-A in antihypertrophic actions in the heart [51–53]. A more detailed analysis of GC-A was performed using mice bearing a conditional knockout of GC-A and indicated the importance of GC-A in vascular endothelial-cell-mediated blood pressure control [54–56].

As for the regulation of ANP and BNP gene expression, neuron-restrictive silencer elements (NRSEs) are located in the 5'-flanking region of the BNP gene and the 3'-untranslated region of the ANP gene [57]. The neuron-restrictive silencer factor (NRSF) can thus repress ANP promoter activity through binding to NRSE [58]. Studies examining dominant-negative NRSF Tg mice expressed under the control of the α -myosin heavy-chain promoter have demonstrated that NRSF plays an important role in the gene expression of both ANP and BNP and in the progression of cardiac dysfunction and lethal arrhythmia associated with heart failure [59].

Genetically engineered mice of the CNP/GC-B system

We generated mice with a targeted disruption of the CNP gene; the resultant CNP-KO mice exhibited markedly short stature due to impaired bone growth [60]. Mammalian bones are formed through two different mechanisms, endochondral ossification and membranous ossification. Most mammalian bones are formed through endochondral ossification, a process during which chondrocytes in the growth plate undergo proliferation, hypertrophy, cell death, and osteoblastic replacement [61]. The short-stature phenotype of CNP-KO mice resulted from impaired bone growth through endochondral ossification [60]. CNP-Tg mice with targeted overexpression of CNP at the growth plate cartilage exhibited prominent overgrowth of those bones formed through endochondral ossification [62]. GC-B-KO mice exhibit the same short-stature phenotype as observed in CNP-KO mice [63], demonstrating that the CNP/GC-B system is a physiologically important stimulator of endochondral bone growth. Dominant-negative GC-B transgenic rats displayed blood-pressure-independent cardiac hypertrophy, suggesting evidence linking GC-B signaling to the control of cardiac growth [64].

cGMP-dependent protein kinase (cGK) has been identified as a molecule activated downstream of the natriuretic peptide family and GC system [65]. Mice depleted with the gene of

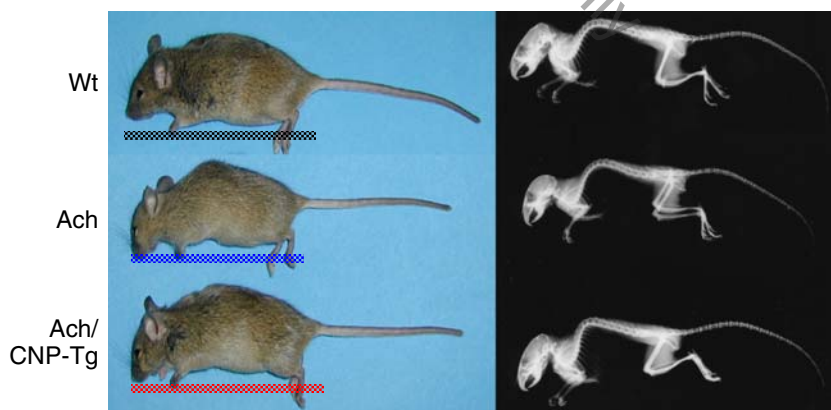
one subtype of cGK, cGKII (cGKII-KO mice), exhibit a short-stature phenotype secondary to impaired endochondral bone growth [66], similar to that observed in CNP-KO mice [60]. We demonstrated that cGKII affected endochondral bone growth by functioning downstream of the CNP/GC-B system by showing that the impaired endochondral bone growth observed in cGKII-KO mice could not be rescued by targeted overexpression of CNP in the growth plate cartilage [67].

Multiple spontaneous animal models with impairments in the CNP/GC-B system have been identified [68–71]. Two strains of dwarf mice, with an autosomal recessive mutant gene, named *cn/cn* [68] and short-limbed dwarfism (SLW) mice [69], possess spontaneous loss-of-function mutations in the *GC-B* gene. Spontaneous mutant mice with a loss-of-function mutation in the CNP gene, named long bone abnormality (Lbab) mice, exhibit short-stature owing to their impaired endochondral bone growth [70], and this phenotype could be abrogated by targeted overexpression of CNP in the growth plate cartilage [71].

Clinical application of CNP and its analogs for skeletal dysplasia

To explore the potential applications of CNP and its analogs for clinical use, we attempted to apply the strong effect of CNP and GC-B on endochondral bone growth to skeletal dysplasia, a group of genetic disorders characterized by severely impaired bone growth [72]. Achondroplasia (Ach), the most common form of skeletal dysplasia characterized by short-limbed dwarfism, is caused by constitutive activation of fibroblast growth factor (FGF) receptor 3 [73]. The current therapy for Ach is limited to distraction osteogenesis [74], an orthopedic procedure; no efficient medical therapies have been developed as yet. We demonstrated that targeted overexpression of a CNP transgene in the growth plate cartilage of a mouse model of achondroplasia (Ach mice) rescues their impaired bone growth and short-stature phenotypes [62] (Fig. 1). To elucidate the molecular

Fig. 1 Rescue of achondroplastic mice (Ach mouse) by targeted overexpression of CNP in growth plate cartilage. From top to bottom are shown the gross appearance (left panel) and skeletal phenotype (right panel, soft X-ray picture) of female wild-type mice (*Wt*), Ach mice (*Ach*), and Ach mice overexpressing CNP in the growth plate cartilage (*Ach/CNP-Tg*) at an age of 3 months



mechanism by which CNP ameliorates achondroplasia, we examined the effect of CNP on extracellular signal-regulated kinase (ERK) signaling. CNP inhibited FGF2-stimulated phosphorylation of ERK in a dose-dependent manner through cGMP activation via GC-B ligation, ultimately increasing matrix synthesis by chondrocytes [62].

We also demonstrated that systemic and continuous administration of synthetic CNP is safe and effective to reverse the impaired bone growth seen in Ach mice [75] (Fig. 2). The safety and efficacy of systemic CNP administration in preclinical studies with the observation that CNP has only a minimal effect of blood pressure in humans [76] suggest that systemic administration of CNP or CNP analogs provides a novel therapeutic strategy for the treatment of human skeletal dysplasia, including Ach.

One form of human skeletal dysplasia, acromesomelic dysplasia type Maroteaux, is caused by loss-of-function mutations in the GC-B gene [77]. This implicates the CNP/GC-B system as a physiologically important enhancer of endochondral bone growth in humans, suggesting a clinical application for CNP and CNP analogs to multiple types of human skeletal dysplasia [75].

In the near future, idiopathic short stature, a common disease of short-stature phenotype with an unknown etiology, and bone fracture, the healing of which is made through endochondral ossification, would be the next avenues to explore for a therapeutic effect of CNP treatment.

Translational research of leptin

Leptin, an adipocyte-derived hormone originally identified from hereditary obese mice (*ob/ob* mice) [78], plays crucial physiologic roles in the regulation of energy expenditure and food intake [79–83]. Mice [84] and rats [85, 86]

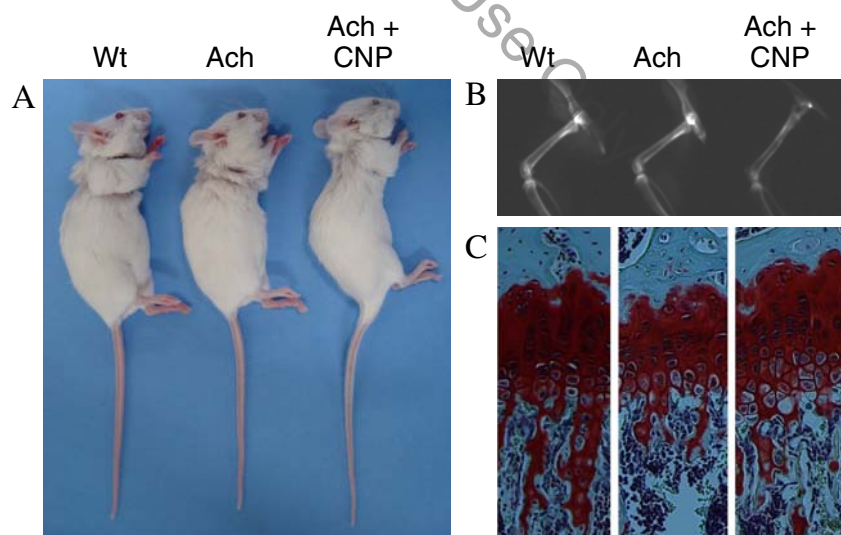
bearing mutations in leptin receptors demonstrate identical phenotypes as *ob/ob* mice. The Koletsky rat, an obese substrain of SHR serving as a model of metabolic syndrome exhibiting both hypertension and morbid obesity, was discovered to carry an additional nonsense mutation of the leptin receptor [86].

In obese animals and subjects, plasma leptin concentrations are increased in proportion to the degree of adiposity [87–89], indicating that leptin is a satiety signal communicating the size of adipose stores to the brain [90–92] and that leptin resistance is related to obesity [87, 93–95]. Leptin deficiency in human subjects is associated with morbid obesity with insulin resistance, indicating the physiological role of leptin in both animal models and humans [96, 97]. Leptin is implicated in a number of manifestations seen in obese animal models [91, 98–101], especially obesity-related hypertension [99], abnormal reproduction [98], bone changes [100], and Cushing syndrome [102]. Leptin is also produced by human placenta [103] and choriodecidual tumors [104].

Generation of Tg mice overexpressing leptin

To explore the clinical implications of leptin *in vivo*, we generated leptin-Tg mice displaying elevated plasma leptin concentrations comparable to those seen in obese subjects [105]. A fusion gene comprised of the human SAP promoter upstream of the mouse leptin cDNA coding sequences was designed to target hormone expression to the liver [43, 106]. Overexpression of leptin in the liver resulted in the complete disappearance of both white and brown adipose tissues in mice [105]. Such a phenotype did not occur when transgene expression was targeted to adipose tissue, the endogenous site of leptin production, using adipocyte-specific promoters [107]. The hyperleptin-

Fig. 2 Rescue of Ach mice by administration of synthetic CNP. Three-week-old female wild-type (*Wt*) or Ach mice were continuously administered CNP intravenously. The gross appearances (a), soft X-ray pictures of femurs (b), and histological pictures of tibial growth plates stained with safranin-O and hematoxylin and eosin (c) are shown for wild-type mice treated with vehicle (left), Ach mice treated with vehicle (middle), and Ach mice treated with 1 $\mu\text{g}/\text{kg}$ per minute CNP (right) after a 4-week administration period. Scale bar in c, 50 μm



nemia seen in these transgenic “skinny” mice provides a unique experimental system in which the long-term effects of leptin are investigated *in vivo* [98–101, 105, 108, 109]. Skinny mice exhibit augmented glucose metabolism and increased insulin sensitivity of both skeletal muscle and liver [105], supporting the concept that leptin acts as an antidiabetic hormone *in vivo* [110–112]. These studies suggest the potential usefulness for leptin treatment of diabetes and obesity.

Crossbreeding of transgenic skinny mice with A-ZIP/F-1 mice, a mouse model of severe lipotrophic diabetes

Generalized lipodystrophy, caused by a systemic deficiency of adipose tissue, is characterized by severe insulin resistance and hypertriglyceridemia [113]. A form of diabetes, called lipotrophic diabetes, eventually develops, although the precise mechanism by which this paucity of fat results in diabetes has remained to be elucidated. Plasma leptin concentrations are markedly reduced or absent in patients with lipotrophic diabetes and in rodent models of this disease [114–117]. Given leptin’s antidiabetic action, leptin deficiency may play a role in the pathogenesis of lipotrophic diabetes; thus, leptin may be a drug for lipotrophic diabetes.

A mouse model of severe lipotrophic diabetes (A-ZIP/F-1) was generated by expressing in adipose tissue a protein that inactivates basic-zipper transcription factors [116]. To assess the pathophysiological role and therapeutic potential of leptin in lipotrophic diabetes, we crossed transgenic skinny (LepTg⁺) and A-ZIP/F-1 (A-ZIPTg⁺) mice to produce double transgenic mice (LepTg⁺:A-ZIPTg⁺) virtually lacking adipose tissue and expressing approximately tenfold higher levels of leptin than normal controls [118]. LepTg⁺:A-ZIPTg⁺ mice were hypophagic in comparison to A-ZIPTg⁺ mice and exhibited decreased hepatic steatosis. Glucose and insulin tolerance tests displayed increased insulin sensitivity and normal glucose tolerance in LepTg⁺:A-ZIPTg⁺ mice, which was comparable to LepTg⁺ mice. Pair-feeding experiments demon-

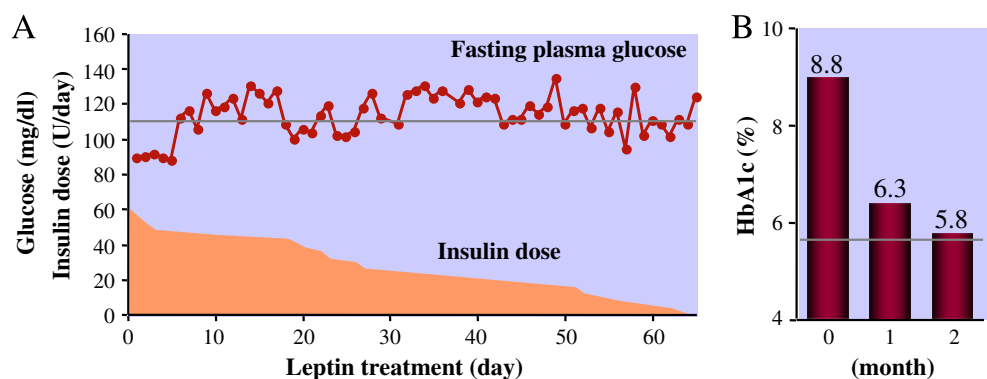
strated that the effects of leptin were not solely due to decreased food intake. Leptin also helped to prevent diabetic nephropathy in generalized lipotrophic diabetes mice [101]. These results demonstrate that leptin can improve insulin resistance and diabetic manifestations in a mouse model of severe systemic lipodystrophy, indicating that leptin is therapeutically useful in the treatment of lipotrophic diabetes [118].

Leptin replacement therapy in Japanese patients with generalized lipodystrophy

We previously reported a novel homozygous mutation of *MC4R* in a Japanese woman with severe obesity (body mass index (BMI) 62 kg/m²) [119]. *MC4R* mutations have been identified at a relatively high frequency (3–4%) in morbidly obese patients in Europe; all of the mutations reported to date occur in an autosomal-dominant fashion, with the exception of a single unique pedigree in the UK. [120, 121]. Although both parents were heterozygous for the mutation, neither exhibited such a severe obese phenotype (BMI 27 and 26 kg/m², respectively, which are preobese according to WHO criteria). As genetic backgrounds and lifestyles vary significantly between European and Asian countries, it is necessary to examine the effect of lifestyle on the phenotypes resulting from genetic mutations and on treatment efficacy in each country.

Four-month leptin replacement therapy has been reported to improve glucose and lipid metabolism in lipodystrophy patients in the USA [122]. To elucidate the efficacy, safety, and mechanisms underlying leptin replacement therapy in Asian patients with generalized lipodystrophy, we treated seven Japanese patients, two acquired and five congenital types, with physiological replacement dose of leptin [123, 124]. Leptin replacement therapy dramatically improved fasting glucose (mean±SE, 172±20 to 120±12 mg/dl, $P<0.05$) and triglyceride (mean±SE, 700±272 to 260±98 mg/dl, $P<0.05$) levels within 1 week. Leptin replacement reduced insulin resistance, as demonstrated by the euglycemic clamp method. Improvement of

Fig. 3 **a** Daily insulin doses and fasting plasma glucose levels and **b** HbA1c levels during the first 2 months of leptin therapy in a 19-year-old male patient with congenital generalized lipodystrophy (Seipin gene mutant)



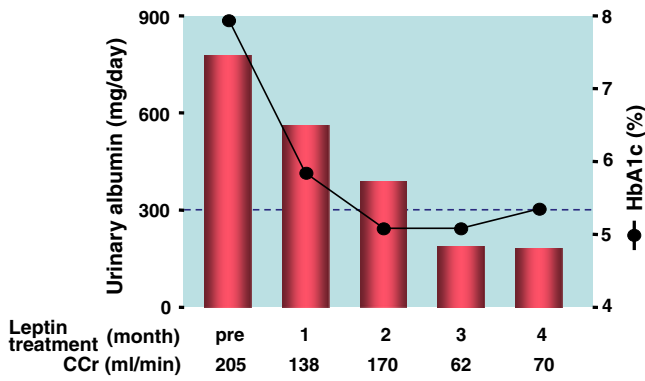


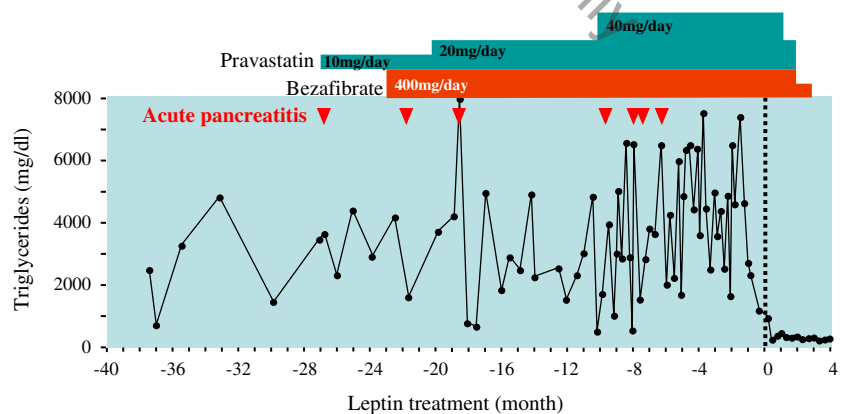
Fig. 4 Time course of daily urinary albumin secretion, creatinine clearance, and HbA1c levels during leptin treatment of a 16-year-old female patient with acquired generalized lipodystrophy

fatty liver was also confirmed by changes in computed tomography (CT) attenuation, and liver volume was calculated by CT imaging. By 4 months, six of seven patients were able to discontinue all antidiabetic drugs, including insulin (Fig. 3). The decreased fasting plasma glucose levels, triglyceride levels, and liver volumes in all seven patients were well maintained throughout the therapy period with no adverse effects. The longest period of leptin replacement therapy has now extended beyond 7 years.

Leptin treatment was also effective at combating diabetic complications. The macroalbuminuria seen in two patients regressed to microalbuminuria, while microalbuminuria in two additional patients normalized. The creatinine clearance of patients with glomerular hyperfiltration decreased with improved glucose tolerance (Fig. 4), which was consistent with previous findings in the lipotrophic diabetes model mice [101].

We also examined the effect of leptin therapy on a 16-year-old girl with severe hypertriglyceridemia who suffered from repeated episodes of acute pancreatitis (Fig. 5). After the initiation of leptin therapy, her triglyceride levels normalized; she did not have any additional episodes of acute pancreatitis (Fig. 5). These results clearly demonstrate

Fig. 5 Fasting serum triglyceride levels, doses of lipid-lowering drugs, and episodes of acute pancreatitis (red inverted triangle) before and after leptin therapy in a 16-year-old girl with acquired generalized lipodystrophy



the safety and efficacy of the long-term leptin replacement therapy in patients with generalized lipodystrophy. While these results are impressive, it is important to remember that the efficacy of leptin replacement therapy in patients from Japan, a country in which the prevalence of obesity is relatively low, is excellent.

Leptin therapy for more prevalent forms of diabetes

To assess the therapeutic potential for leptin treatment in insulin-deficient diabetes, we generated diabetic animals by treating wild-type and LepTg/+ mice with a relatively low dose of streptozotocin (STZ 180 g/g body weight) [125]. Plasma insulin concentrations were reduced (<0.10 ng/ml), resulting in severe hyperglycemia in both wild-type and LepTg/+ mice 2 weeks after STZ treatment. LepTg/+ mice were more sensitive to exogenously administered insulin than wild-type mice; STZ-treated LepTg/+ mice became normoglycemic at doses of insulin that did not improve the hyperglycemia in STZ-treated wild-type mice. To clarify if combination therapy with leptin and insulin is beneficial for insulin-deficient diabetes, we also examined the effect of chronic coadministration of leptin and insulin in STZ-treated wild-type mice. We demonstrated that subthreshold doses of insulin, which do not affect glucose homeostasis, are effective at improving diabetes in STZ-treated wild-type mice in combination with leptin. These results indicate that leptin therapy may be used as an adjunct for insulin therapy in insulin-deficient diabetes.

We also investigated the therapeutic usefulness of leptin in a mouse model of type 2 diabetes mellitus with increased adiposity [126], generated using a combination of a low-dose STZ (120-g/g body weight) and a high-fat diet (HFD, 45% of energy as fat; STZ/HFD). In STZ/HFD mice, continuous infusion of leptin (20-ng/g body weight per hour) reduced food intake and body weight gain and improved glucose and lipid metabolism with enhanced insulin sensitivity. Leptin therapy also decreased the triglyceride content of both the liver and skeletal muscle.

These results indicate a beneficial effect of leptin therapy for type 2 diabetes mellitus with increased adiposity, which corresponds to a BMI in the range of 25–30 kg/m² [126].

Our previous and ongoing studies utilizing transgenic skinny mice and other animal models have demonstrated the pleiotropic actions of leptin in the regulation of energy homeostasis and food intake [98–101, 105, 108, 109] and its clinical usefulness as a therapy for multiple conditions, particularly diabetes mellitus [108, 118, 124, 125]. Tg skinny mouse may be a useful model to study the long-term effects of leptin therapy in vivo and to evaluate the clinical implications of leptin therapy.

Conclusions

Currently, the primary targets of our ongoing translational research of CNP and leptin are achondroplasia and lipotrophic diabetes, respectively. Demonstration of the efficacy of CNP therapy for achondroplasia and leptin replacement therapy for lipotrophic diabetes has relied heavily on basic and preclinical studies using excellent animal models. Although lipotrophic diabetes is a rare disease in humans, the safety and efficacy of leptin replacement therapy for patients with lipotrophic diabetes have been well established. Achondroplasia, while also a rare disease in humans, may be effectively managed with CNP therapy.

It has been possible to establish the safety and efficacy of these hormones in rare human diseases through studies that began with excellent animal models. These studies provided us with novel treatments for common human diseases, which were explored as adjacent to or in extension of these rare human diseases, as seen in the study of hypertension. Research on the SHR animal model and study of a relatively rare cause of hypertension, renovascular hypertension, led to more detailed studies on the blockade of renin–angiotensin system, bringing research forward to the current widespread field of cardiovascular disorders in translational research. These lessons teach us the importance of the breakthroughs using animal models and rare human diseases.

Conflict of interest statement The authors declare that they have no conflict of interests.

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A novel mechanism for inflammation-associated carcinogenesis; an important role of activation-induced cytidine deaminase (AID) in mutation induction

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Abstract Inflammation is a risk for cancer development; however, its mechanism is unknown. Recent studies have revealed that activation-induced cytidine deaminase (AID), which plays essential roles in both class-switch recombination and somatic hypermutation of immunoglobulin gene in B lymphocytes, is aberrantly expressed in non-lymphoid cells not only by *H.pylori* and HCV infection but also by various proinflammatory cytokines, leading to the generation of gene mutations. These findings not only suggested a new mechanism of inflammation-associated carcinogenesis but has also opened up a new field of tumor biology.

Keywords Activation-induced cytidine deaminase (AID) · Inflammation · Carcinogenesis · *Helicobacter pylori* · Gene mutation

Introduction

Since Virchow's era, a causal relationship between inflammation and cancer development has been proposed in a variety of chronic inflammatory diseases. In particular, many cancers of digestive organs, some of which are caused by infectious agents, are known to arise on a background of chronic inflammation [1, 2]. These include *Helicobacter pylori* (*H. pylori*)-induced gastric cancer, hepatitis C virus (HCV)- and hepatitis B virus (HBV)-related hepatocellular carcinoma,

Barrett's esophageal adenocarcinoma, colitis-associated colon cancer and cholangiocarcinoma accompanied by primary sclerosing cholangitis (PSC) [2]. There are many pathways that can lead to cancer development through inflammation. First, microorganisms such as *H.pylori*, HBV and HCV may directly modulate cellular function, giving the cells growth advantages and resistance to apoptosis [3, 4]. Inflammation also induces many mediators and cellular effectors that appear to be involved in carcinogenesis. These include various cytokines, chemokines and growth factors [2, 5, 6]. Moreover, cyclooxygenase 2 (COX2) produced in inflammatory condition is known to enhance tumorigenesis through various mechanisms [7]. In addition, reactive oxygen species (ROS) induced during inflammation have been shown to have mutagenic activity [8].

On the other hand, mutation is recognized as a hallmark of cancer. Indeed, cancers are derived from a clonal proliferation of the transformed cells caused by accumulation of various genetic alterations in proto-oncogenes, tumor-suppressor genes, and other genes that control cell proliferation, regeneration, and apoptosis. Supporting this idea, recent studies analyzing a large number of genomes in human cancers have revealed that a single cancer cell generally possesses approximately 70–90 mutations, 10–15 of which are so-called “driver genes” that contribute to cancer development [9]. Because normal mutation rates cannot account for such multiple mutations in cancer cells, certain molecular mechanisms must be present to explain a large number of nucleotide alterations. One mechanism for the enhanced susceptibility to mutagenesis may be a defect in DNA repair systems. Indeed, dysfunction of the mismatch repair system results in familial colorectal cancer syndrome, and defects in the nucleotide excision repair system are associated with colon and skin cancer [10, 11]. However, the frequency of such defects in the DNA repair system is

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generally low in human cancers. Considering these facts, it is tempting to hypothesize that inflammation may trigger gene alterations during carcinogenesis.

Previous studies have identified many exogenous mutagenic agents, and several possible intrinsic mutagens have also been proposed. Among these, ROS produced during inflammation are known to elicit mutations, particularly G to T transversions [11]. However, a recent study has shown that G to T transversion accounts for only a minor proportion of the total mutations in human cancer cells, and instead C/G to T/A transition is the most prevalent mutation especially in gastrointestinal malignancies [12]. Thus, alternative mechanisms for mutagenesis during inflammation-associated carcinogenesis should be considered.

Activation-induced cytidine deaminase (AID) in B lymphocytes is a physiologic genome mutator essential for somatic hypermutation of immunoglobulin genes

Generally, cells have several systems to prevent mutations so as to avoid harmful nucleotide alterations, so-called “somatic mutations”. However, there is one type of cell in which somatic mutations frequently occur under physiological condition. That cell type is B lymphocytes in which immunoglobulin genes undergo somatic hypermutations to generate molecular diversity against many antigens. Prof. Honjo’s group at Kyoto University first cloned the gene responsible for immunoglobulin class switch recombination in 1999 [13]. They found that this gene was closely related to apolipoprotein B RNA-editing cytidine deaminase 1, which converts cytosine nucleotides to uracils in RNA, and named it activation-induced cytidine deaminase (AID). Interestingly, they subsequently found that this molecule is also responsible for somatic hypermutation of immunoglobulin genes [14]. Notably, AID is the only human enzyme known to induce DNA mutation in human genomes, although under normal conditions it is expressed only in B cells. AID theoretically induces C/G to T/A transitions by its cytidine deaminase activity. In this regard, it should be emphasized that a recent report on systemic sequencing of cancer genomes clearly demonstrated that the most prevalent mutation pattern in human cancers is C/G to T/A transition, a pattern similar to that induced by AID [12].

AID transgenic mice develop not only lymphomas but also various types of cancers

After cloning of the AID gene, many investigators have found overexpression of AID in human lymphoid malignancies, suggesting involvement of AID in human lymphomagenesis [15, 16]. Prof. Honjo’s group established AID transgenic

mice and, as expected, nearly 100% of these developed lymphomas, again suggesting roles of AID in lymphomagenesis, probably by inducing a range of mutations [17]. We then wanted to see whether these mice also developed cancers, because they expressed AID not only in lymphocytes but also in other cells including epithelial cells. Interestingly, it was found that in addition to lymphomas these mice developed many types of cancers, including lung, liver, and gastric cancers, and cholangiocarcinomas [17–19]. These observations prompted us to speculate that AID may be involved in human carcinogenesis, though expression of AID had been believed to be strictly restricted to B cells under normal conditions.

AID is strongly expressed in HCV-infected liver and liver cancers and in *H.pylori*-infected gastritis mucosa and gastric cancers

We, therefore, first examined whether AID is expressed in clinical specimens of liver tissues of patients with HCV infection, and surprisingly we were able to show by immunohistochemistry strong expression of AID not only in liver cancers but also in chronic hepatitis tissues [20, 21]. AID expression was also found in both chronic gastritis mucosa and gastric cancer tissues of *H.pylori*-infected patients, and eradication of *H.pylori* could reduce its expression [19, 22]. Later, aberrant AID expression was also revealed in cholangioepithelium of patients with PSC [23], and in colonic mucosa and cancer tissues of patients with inflammatory bowel disease, but not in normal colonic mucosa [24]. All these data strongly suggested the involvement of AID in inflammation-associated carcinogenesis in human.

AID is induced via NFκB activation by *H.pylori* and HCV core protein, and causes gene mutations

We next examined AID expression in human hepatocytes and gastric cells *in vitro*, and tried to elucidate the mechanisms for AID expression in non-lymphoid cells. We first observed that AID is induced by expression of HCV core protein or by *H.pylori* infection. Then, because AID expression in B cells was known to be induced by NFκB activation through CD40 ligation by T cells, and because both *H.pylori* and the core protein of HCV enhance NFκB activation [4, 25], the roles of NFκB in AID expression in epithelial cells were examined. It was found that introduction of the gene for the core protein of HCV into human hepatocytes induced AID expression via NFκB activation [20]. Induction of AID expression in human gastric cells by *H.pylori* infection was also found to be dependent on NFκB [19]. Because *H.pylori* deficient for Cag pathogenicity island (PAI) completely lost

its ability to induce both NFκB activation and AID expression [19], it was considered that certain *H.pylori* factors that are introduced into epithelial cells through the *H.pylori* type IV secretion machinery could cause AID expression via NFκB activation. In this regard, Viala et al. [26] reported that *H.pylori*-derived peptidoglycan introduced via the type IV secretion apparatus is responsible for NFκB activation. We further observed *in vitro* that *H.pylori* infection resulted in mutations in various genes including *p53*, which could be inhibited by knockdown of endogenous AID using AID siRNA [19]. Taken together, the following scenario may be illustrated: both *H.pylori* and HCV infection generate gene mutations by inducing AID expression through NFκB activation (Fig. 1).

In addition to *H.pylori* and HCV core protein themselves, AID expression is also induced by IL1β and TNFα through NFκB activation, by IL4 and IL13 through STAT6, and by TGFβ[20, 24]. These cytokines are known to enhance AID expression in B lymphocytes. Thus, these cytokines appear to be involved in inflammation-associated cancer development by accelerating gene mutations (Fig. 2).

Lessons for cancer research in the study of AID

Roles of NFκB in carcinogenesis

Recent cancer studies have focused on the important roles of NFκB in inflammation-associated carcinogenesis. Indeed, NFκB is activated not only by microorganisms but also by many cytokines. However, most investigators are interested in its growth-promoting and anti-apoptotic activity, and in its role in enhancing inflammation during cancer development.

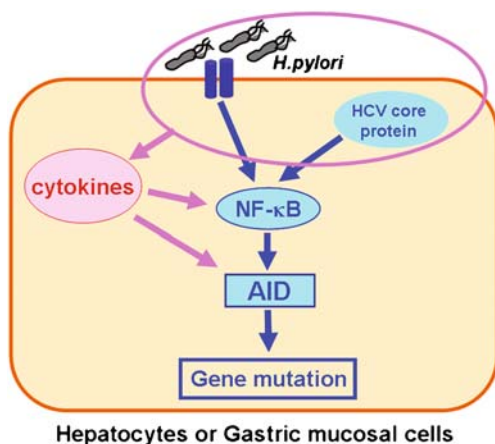


Fig. 1 Induction of AID and gene mutations by *H.pylori* and HCV infection. Certain *H.pylori* factors that are introduced into epithelial cells through the *H.pylori* type IV secretion machinery and HCV core protein could cause AID expression via NFκB activation. *H.pylori* and HCV can also induce AID expression indirectly by enhancing various cytokines production

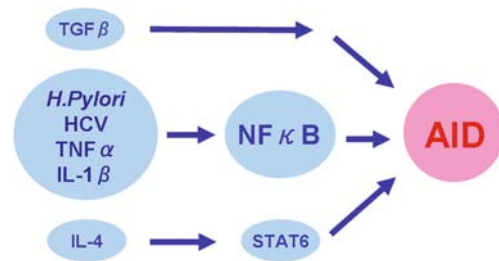


Fig. 2 Induction of AID by various cytokines. In addition to *H.pylori* and HCV core protein themselves, various cytokines induced by inflammation can also elicit AID expression

In contrast, our studies demonstrated that NFκB activation is involved in generating gene mutation by inducing AID expression, which may suggest a new role for NFκB in inflammation-associated carcinogenesis (Fig. 3).

Different spectra of gene mutations in different cancers

With the exception of *p53*, the cancer genes that are frequently mutated differ for different types of cancers. For instance, *k-ras* mutations are found in the majority of the pancreatic cancers whereas only a few gastric cancers have *k-ras* mutations [27]. Moreover, although *apc* mutations are rarely found in hepatocellular carcinomas, many colon cancers possess this mutation. The reason for such distinct mutation patterns in different cancers is not clear. Because the importance of each gene as a tumor suppressor or as a tumor enhancer may be different in different cell types such as pancreatic cells, gastric cells and colonocytes, we may be able to find distinct spectra of mutated genes in cancer cells that have arisen from different tissues. In this context, it is interesting to note that the genes targeted by AID are different in different cells [18]. Indeed, induction of AID causes mutations at *p53* and *βcatenin* but not at *c-myc* in gastric cells, whereas it induces *c-myc* mutations in hep-

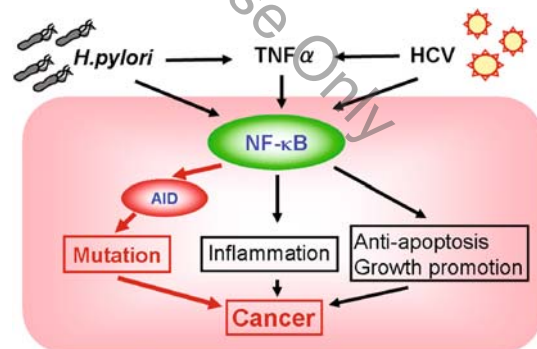


Fig. 3 Important roles for NFκB in inflammation-associated carcinogenesis. NFκB is known to play roles in inflammation-associated carcinogenesis by further enhancing inflammation and also through its anti-apoptotic or growth promoting action. Moreover, NFκB contributes to inflammation-associated carcinogenesis by enhancing gene mutations through AID induction

atocytes and lymphocytes [19–21]. In the case of immunoglobulin gene somatic hypermutation in B lymphocytes, AID recognizes a consensus sequence in the immunoglobulin gene to induce mutations. However, in addition to the immunoglobulin gene, *Bcl6* is known to be an excellent target for AID, for reasons that are not clear at present. Unlike the immunoglobulin genes, we have so far been unable to find any consensus sequence in various cancer-related genes that is recognized by AID. One mechanism may be that AID targets genes that are undergoing active transcription [28]; however, it is clear that other mechanisms by which AID recognizes specific target genes are also present. In any case, it is interesting to speculate that AID is involved in the development of distinct mutation patterns in different cancers.

Relationship between mutation induction and repair systems

As already mentioned, AID deaminates cytosine to produce uracil and, after DNA replication, the paired guanine is mutated to adenine. Then, after further DNA replication, the original cytosine is mutated to thymidine, and thus eventually a C/G to T/A transition develops. The importance of the induction of C/G to T/A mutation by AID may be supported by the fact that C/G to T/A transitions are the most prevalent mutation found in human GI cancers [12]. However, although C/G to T/A transitions are the most prevalent after AID induction, other mutations also develop [29]. In agreement with these data, a range of mutations for which AID is entirely responsible do occur even in immunoglobulin gene somatic hypermutation. The reason why AID induces different mutations is not completely clear at present. However, it is possible that several repair systems including mismatch repair and excision repair become involved after cytosine deamination by AID, and it is important to note that these repair systems do not always provide high-fidelity repair [30, 31]. For instance, when excision repair is initiated by uracil endoglycosidase before DNA replication, an abasic site is produced. When DNA replication starts in such a situation, various mutations may ultimately occur because of the involvement of error-prone DNA polymerases with relatively low fidelity. Thus, the ultimate mutation spectrum may be determined by a balance between the mutagenic activity of AID and the involvement of repair systems [32].

Possible role for AID in genomic instability

Recent studies have shown that, in addition to mutation induction, AID is also responsible for chromosomal translocations through production of double-strand DNA breaks in the development of B cell malignancies. Indeed, Nussenzweig et al. [33, 34] reported that AID is required for chromosomal breaks in not only *IgH* but also *c-myc* that lead to *c-myc/IgH*

translocations. In this connection, cancer cells possess a considerable number of gene duplications and deletions. Because gene deletion requires DNA breaks and AID can induce DNA breaks, it may be reasonable to consider that ectopically-expressed AID is involved in not only gene mutations but also gene deletions in cancer cells. Thus, whether AID is able to explain accumulation of both mutations and deletions of genes during carcinogenesis is an interesting question to be clarified in future studies.

Summary

The finding that AID expression is induced in non-lymphoid cells in various inflammatory conditions has not only proposed a new mechanism of inflammation-associated carcinogenesis but has also opened up a new field of tumor biology. Recent studies have suggested the importance of both genetic and epigenetic changes in cancer development, and the relative importance of each is always a matter of discussion. Under such circumstances, we believe that the study of AID is able to “reinforce” cancer genetics. Moreover, a very recent study has demonstrated that AID is involved in programmed DNA demethylation of the zebrafish genome during embryonic development. Thus, it is tempting to speculate that AID plays important roles in linking between genetics and epigenetics [35].

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