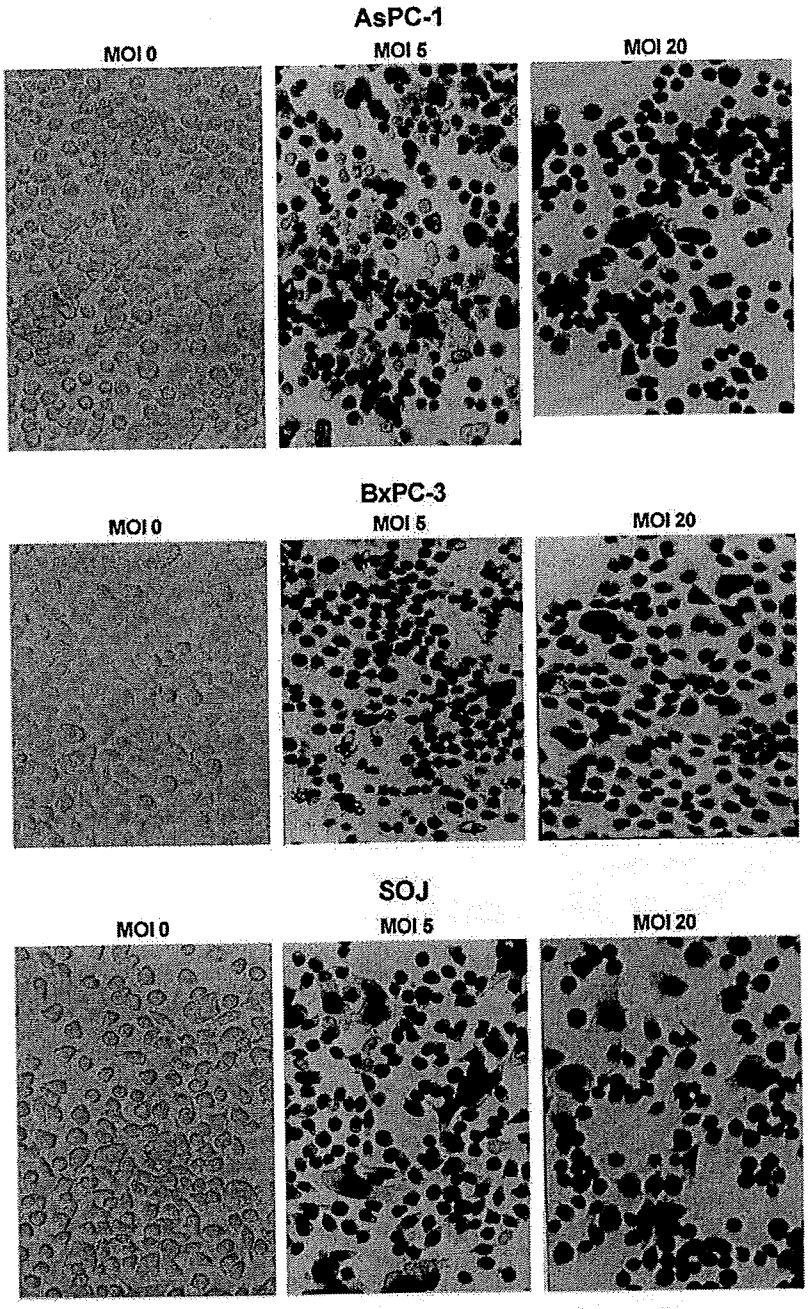

Fig. 2. Transduction efficiency of adenovirus in cancer cells *in vitro*. Optimal MOI for adenovirus-mediated gene transduction without virulence was determined in pancreatic cancer cells with control viral vector AdCA*lacZ*. AsPC-1, BxPC-3 and SOJ cells were plated on 24-well plates and transfected with AdCA*lacZ* at MOI of 0, 5, and 20. Forty-eight hours later, cells were fixed and stained with x-gal to demonstrate *lacZ* gene expression. The magnification of all photographs is ×400. No stained cells were detected at MOI 0 in the left panels, and increased transduction of *lacZ* in the right two panels of each cell line.

4. Discussion

The development of gene transfer technologies has provided new possibilities for the treatment of malignancies. Adenovirus vector systems that can produce high titers of viruses capable of efficient expression in target cells can deliver exogenous genes into a variety of cells and tissues.

Matsuda et al. reported that 185 kDa protein immunoreactive to anti-erbB-2 antibodies was detected in TOB immunoprecipitates, and a 45 kDa protein reactive to the anti-TOB antibodies was co-immunoprecipitated with $p185^{erbB-2}$, reciprocally [6,7]. TOB physically interacts with the *c-erbB-2* gene product. Exogenously expressed TOB is able to suppress the growth of NIH3T3 cells, and delivers growth inhibitory signals. These findings raise the possibility that the *tob* is a tumor suppressor gene [6,15]. Inspection of the deduced amino acid sequence of TOB revealed significant homologies (40.6%) to the BTG-1 anti-proliferative gene

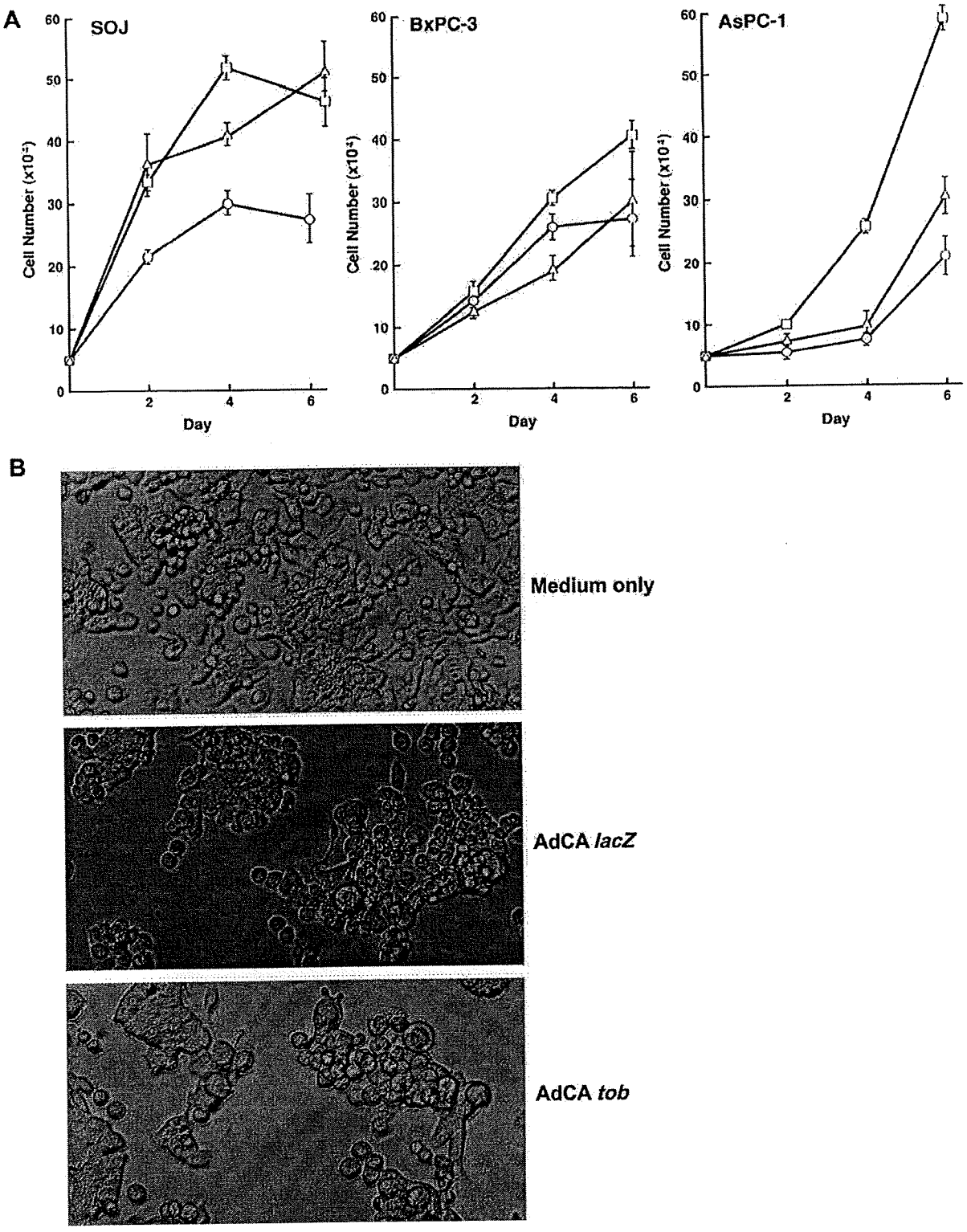

Fig. 3. (A) Tumor suppressor effect of the *tob* gene AsPC-1, BxPC-3, and SOJ cell lines were transfected with AdCA*tob* (○) or AdCA*lacZ* (△) at an MOI of 20. Medium without adenovirus vector (□) is also shown as medium only. These cancer cells could be transfected with MOI 20 without adenoviral virulence. Live cells were counted on the indicated days after transfection. Results are the means ± SD of three independent experiments. (B) Morphologies of transfected AsPC-1 cells with AdCA*tob*, AdCA*lacZ*, medium only without adenovirus vector. Enlarged megakaryotic cells appeared with many granules in cytoplasm. Photograph magnification is ×400.

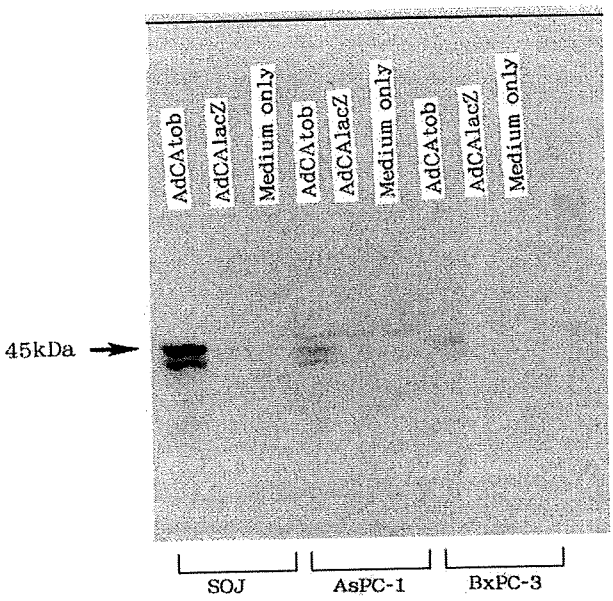

Fig. 4. Western blot analysis of lysates of pancreatic cancer cell lines after transfection of AdCA*lacZ* or AdCA*tob* using the anti-TOB monoclonal antibody Transfected adenovirus vectors are indicated above lanes. Cancer cells are indicated below lanes. Arrow on the left side of gel indicates 45 kDa band specific to exogenous TOB. Band higher than 45 kDa indicates endogenous TOB expressed in AsPC-1 cells.

product and to the PC 3 (BTG-2) gene product at its amino-terminal half. Both BTG-1 and BTG-2, having the significant homologies to TOB, suppress cell growth, and perform cell cycle control [8,9]. ANA, belonging to the anti-proliferative TOB family, interacts with CCR transcription factor-associated protein Caf1 [16,17].

NIH3T3 cells expressing exogenous *tob* mRNA were enlarged and showed swollen nuclei with many granules in the cytoplasm. The characteristics resembled those of senescent cells. Evidence of senescence change were the accumulation of lipofuscin granules, an ultrastructural change associated with aging, and flattened enlarged cell morphology. TOB exhibits anti-proliferative activity when the level of its expression is elevated and/or deregulated. The interaction of TOB with *c-erbB-2* gene products was suggested to occur through the carboxyl-terminal half of TOB. Exogenously expressed TOB also exhibits anti-proliferative activity. Protein-tyrosine kinase receptors induce the expression of G1 cyclins, which in turn interact with and activate CDK family proteins, resulting in the phosphorylation of Rb protein. It is necessary to examine whether protein-tyrosine kinases other than $p185^{erbB-2}$ could also interact with TOB in the proliferative signal transduction of cancer cells. Expression of BTG-1 is high in G0/G1 phases of the cell cycle and is down regulated when the cells enter the growth cycle, suggesting that the gene product is inhibitory to G0/G1 progression. A forced expression of exogenous BTG-1 in NIH3T3 cells resulted in the suppression of cell growth [8]. The BTG-1 gene is 60% homologous to PC3, an immediate early gene induced by nerve growth factor in rat PC12 cells. Rouault et al. named PC3 as BTG-2, and have reported that BTG-2 expression is induced through a p53-dependent mechanism and the function may be relevant to cell cycle control and cellular response to DNA damage [9].

Yoshida et al. had reported that *tob* is a member of anti-proliferative family genes. Mice lacking *tob* are prone to spontaneous formation of tumors. The occurrence rate of diethylnitrosamine-induced liver tumors is higher in $tob^{-/-}$ mice than in wild-type mice. $TOB^{-/-}$ $p53^{-/-}$ mice show accelerated tumor formation in comparison with single null mice. Expression of cyclin D1 mRNA is increased in the absence of and reduced by TOB [18]. Suzuki et al. had reported that TOB inhibits cell growth by suppressing cyclin D1 expression, which is canceled by Erk1- and Erk2-mediated TOB phosphorylation. TOB is critically involved in the control of early G1 progression [19]. Iwanaga et al. reported

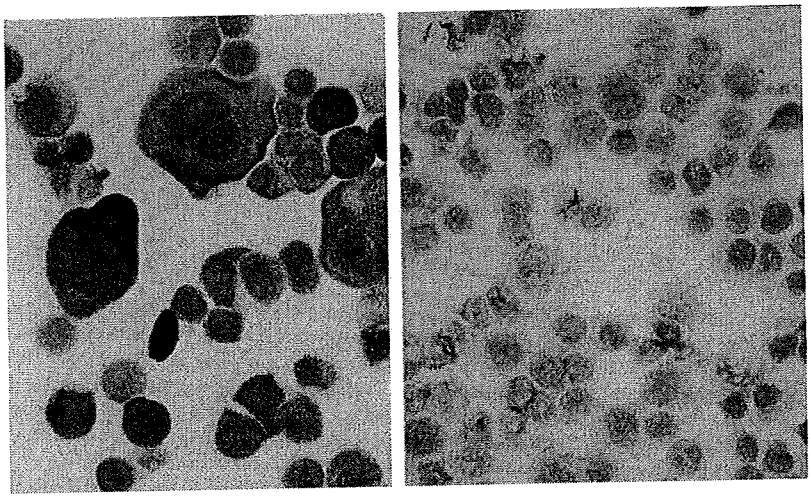

Fig. 5. AdCA*tob* vector-mediated *tob* expression *in vitro* AsPC-1 cells (50×10^3) were transfected with AdCA*tob* vector (MOI 20). After 72 h, cells were collected and stained with anti-TOB monoclonal antibody 4B1 by peroxidase immunostaining in order to determine the expression of TOB protein. Photograph magnification is ×400. Left: transfected cells were well stained. Right: non-treated naïve cells.

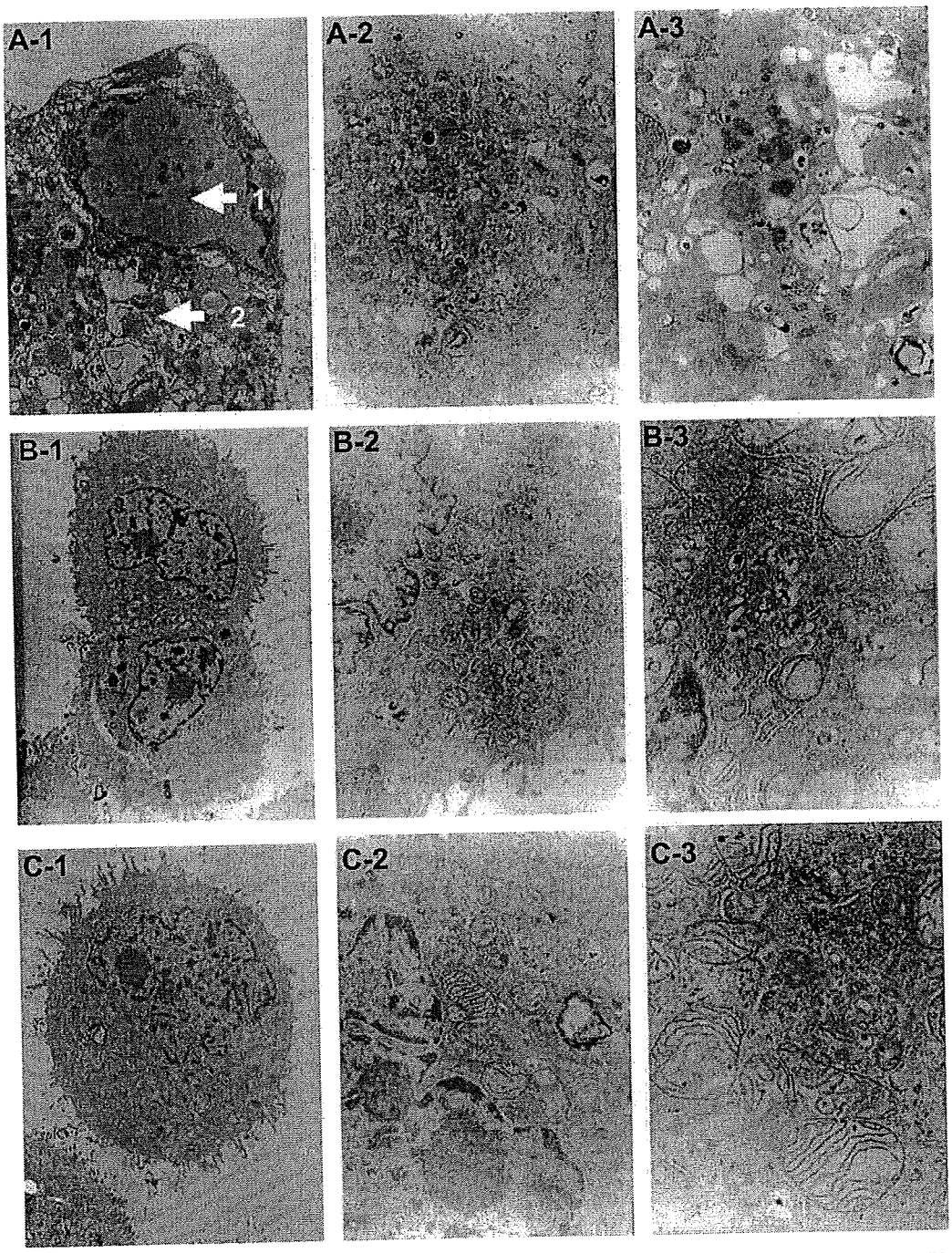

Fig. 6. Cytotoxic effect on AsPC-1 with *tob* expression by AdCA*tob* vector on electron microscopy. (A) AdCA*tob* transfectant (MOI 100), (B) AdCA *mock* transfectant (MOI 100), (C) Non-treated control. (A) AdCA*tob* transfectant (MOI 100) showed degradation of nucleus (arrowhead 1), many autophagosomes and electron-dense cytoplasmic inclusions (arrowhead 2). The contents of these vesicles consisted of lamellar material that resembled lipofuscin, a lipid substance with auto-fluorescence properties. (B) AdCA *mock* transfectant (MOI 100) showed irregular-shaped nucleus, enlargements and deformities of mitochondria, increase of endoplasmic reticulum and lysosome vesicles. No increase of lipofuscin granules. (C) Non-treated control showed irregular-shaped nucleus, enlargement of mitochondria, increase of endoplasmic reticulum. Several autophagosomes were seen in cells treated with AdCA *mock* and non-treated cells, but increase of autophagosomes were recognized in cells treated with AdCA*tob*. There was neither an increase of inside scatter nor the appearance of lipofuscin granules. A-1, B-1, and C-1 are low magnification of the photograph (×5000), A-2, B-2, and C-2 are high magnification of the photograph (×8000), and A-3, B-3, and C-3 are high magnification of the photograph (×15,000).

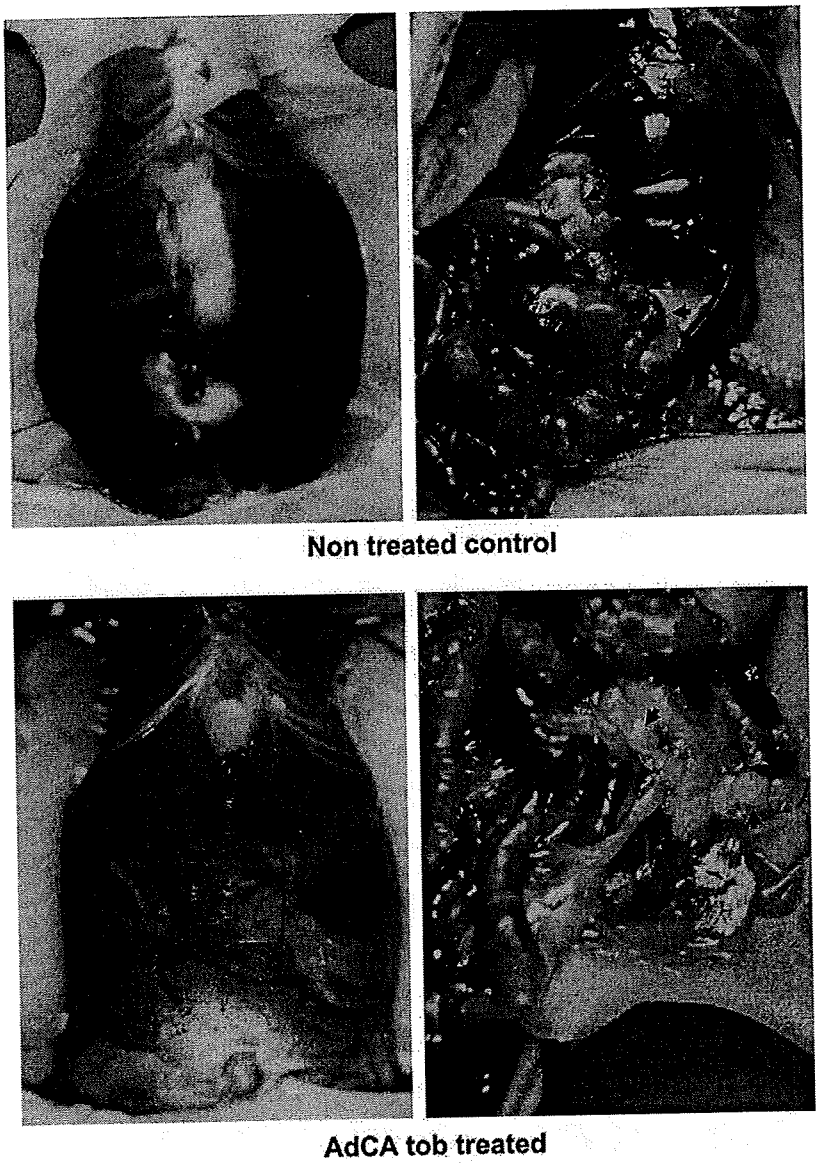

Fig. 7. Tumor growth suppression of cancer peritonitis model with peritoneal AdCA*tob* transfection. Bloody ascites and tumor growth suppression in the abdominal cavity disappeared with viral *tob* expression. (A). Non-treated control; Bloody ascites (Left) and multiple tumor nodules (Right) were seen at the mesentery after 16 days of 2×10^6 ip injection of AsPC-1 cells. (B). AdCA*tob*-treated; mice underwent ip injections of AdCA*tob* (1.5×10^8 pfu/0.2 ml/day, from day 5 to day 9) after 4 days of ip injection of 2×10^6 AsPC-1 cells. Peritoneal injections of AdCA*tob* suppressed tumor nodule formation in the abdominal cavity compared with the non-treated group (Right). Bloody ascites was not seen in AdCA*tob*-treated mice (Left).

that the phosphorylated and inactive form of TOB was detected in 76% of cancer tissues of adenocarcinoma patients, but not in normal alveolar epithelial cells [20]. Cho et al. reported that phosphorylation of myristoylated alanine-rich C kinase substrate, MARCKS, removes TOB from ErbB-2 by increasing its binding affinity with TOB, and thereby activates ErbB-2-mediated signal transduction [21]. TOB phosphorylation contributes to the progression of papillary carcinoma of the thyroid, especially in the later phase through cancellation of its anti-proliferative function [22]. Exogenous overexpression of TOB family proteins suppresses cell proliferation. Mutation in the nuclear localization signal sequence of TOB affects its nuclear localization and impairs anti-proliferative activity [23,24]. ERK phosphorylation negatively regulates the anti-proliferative function of TOB [25]. Sasajima et al. reported that the BTG/TOB family was degraded by the ubiquitin-proteasome system [26].

We evaluated the expression of *tob* mRNA and gene product in pancreatic cancer cell lines, AsPC-1, BxPC-3, and SOJ with or without *tob* transfection. The *tob* mRNA was expressed in all pancreatic cancer cell lines, and the level of *tob* mRNA of AsPC-1 cells was strongest among them. The *tob* mRNA

Table 1
Inhibition of the formation of pancreatic cancer peritonitis with Adeno-virus mediated *tob* gene transfer *in vivo*

Treatment group	Tumor nodules		Ascites	
AdCA*tob* (1.5×10^8 pfu) ($n=4$)	–	(0/4)	±	Serous
AdCA*tob* (5×10^7 pfu) ($n=3$)	+	(1/3)	±	Serous
AdCA*lacZ* (1.5×10^8 pfu) ($n=3$)	+	(2/3)	±	Serous
AdCA*lacZ* (5×10^7 pfu) ($n=3$)	+	(2/3)	+~++	Turbid
Non-treated (1.5×10^8 pfu) ($n=3$)	+++	(3/3)	+++	Bloody

Despite the observed heterogeneity for individual animals, a significant tumor growth inhibitory effects of AdCA*tob* has been noted.

expression was increased in correlation with erbB-2 mRNA expression in AsPC-1 and BxPC-3 cells, but the endogenous *tob* gene product is not increased in these cells.

Overexpression of erbB-2 and EGF-R protein was observed in pancreatic cancer cells, so the suppressive effect of endogenous TOB could be impaired by the passage of protein-tyrosine kinases as $p185^{erbB\text{-}2}$. We prepared adenoviral vector containing *tob* cDNA (AdCA*tob*). AdCA*tob* was transfected and expanded in 293 cells. The titer of the vector was 350×10^6 pfu/ml. Monitoring the viability of pancreatic cancer cells transfected with adenoviral vector containing the *lacZ* gene revealed that these cancer cells were able to be transfected with MOI 20 without adenoviral toxicity. Growth

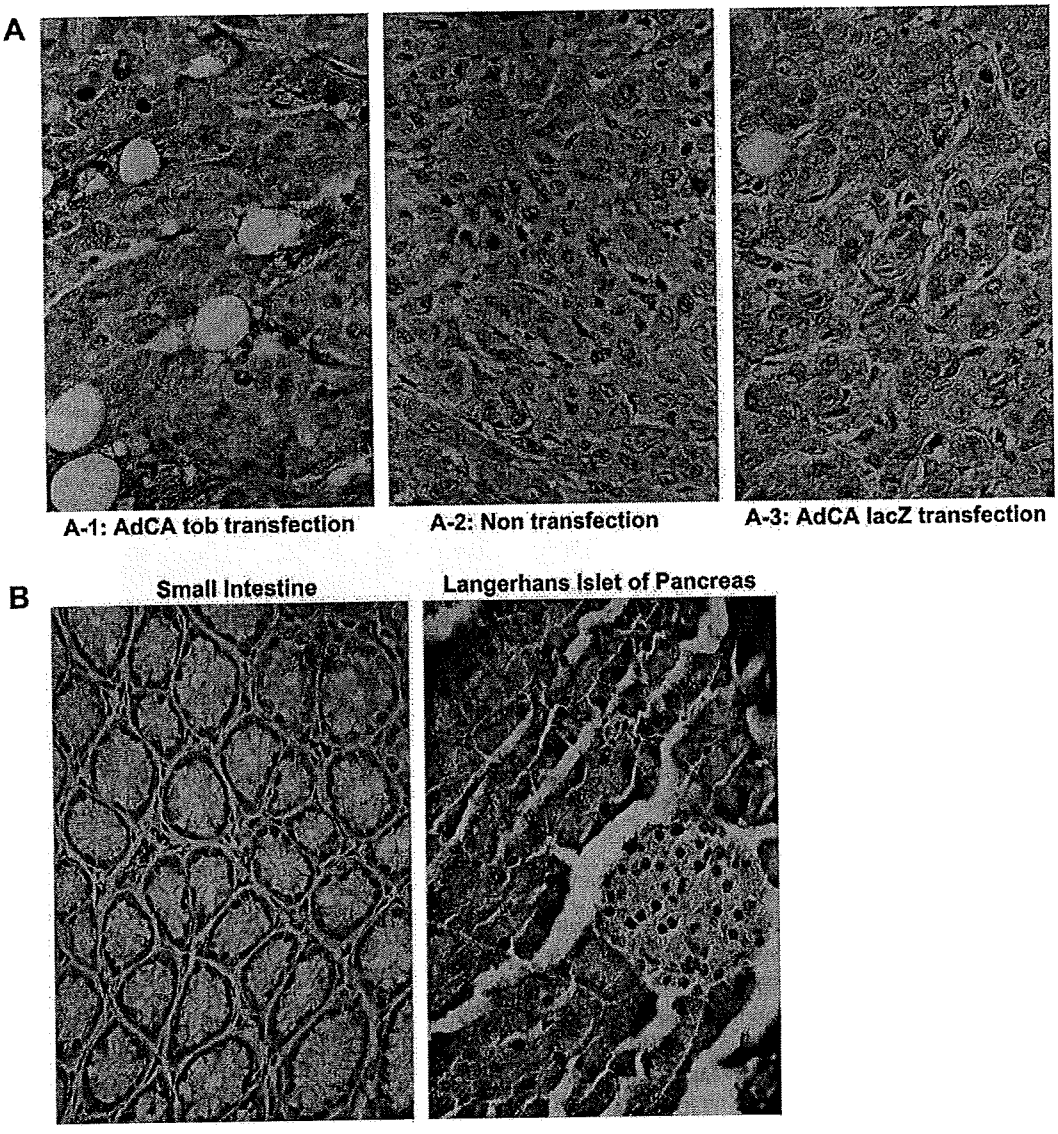

Fig. 8. Pathological findings of tumor treated with AdCA*tob* vector *in vivo*. Only one tumor nodule was recognized in the abdominal cavity in the AdCA*tob* (5×10^7 pfu)-treated group, and not found in 1.5×10^8 pfu groups. The tumor continued to express TOB with staining by anti-TOB monoclonal antibody 4B1 (A-1). Tumors of non-treated control (A-2) and AdCA*lacZ* (A-3)-transfected groups showed only a few endogenous TOB. The jejunum (B-1), mesothelium, acinal gland and pancreatic islets of Langerhans (B-2) did not show the expression of exogenous TOB with AdCA*tob* transfection.

suppression with transfection of AdCA*tob* was shown in SOJ and AsPC-1 cell lines according to *tob* expression. We evaluated the tumor-suppressing effects of AdCA*tob* in pancreatic cancer cell lines. SOJ and AsPC-1 cell lines transfected by AdCA*tob* showed growth suppression. Significant suppression was shown in SOJ cell lines after the overexpression of TOB. These results suggest that the recombinant adenovirus vector containing the *tob* gene is a useful candidate for anti-tumor gene therapy, and could be applied for cancer peritonitis. AsPC-1 cells expressing the exogenous *tob* were enlarged and megakaryocytic with characteristics resembling those of senescent cells.

We also found that TOB-overexpressing AsPC-1 cells showed degradation of the nucleus and many autophagosomes and electron-dense cytoplasmic inclusions. One cancer cytotoxic mechanism is based on autophagy. Autophagy is a cellular degradation pathway for the clearance of damaged or superfluous proteins and organelles [27,28], and a survival pathway required for cellular viability during starvation; however, if it proceeds to completion, autophagy can lead to cell death. Autophagy has emerged as a homeostatic mechanism regulating the turnover of long-lived or damaged proteins and organelles, and buffering metabolic stress under conditions of nutrient deprivation by recycling intracellular constituents. Autophagy is also a form of cell death, when allowed to proceed to excessive levels and when apoptosis-defective cells are triggered to die. It has been thought that autophagy may play an active role in programmed cell death [29]. We had observed autophagic conformation by the formulation of autophagosomes and localization of GFP-LC3 on the cytotoxicity of human pancreatic cancer cells treated with polyoxomolybdates (PM-17) [30]. Faiy et al. indicated that quercetin induced autophagy specifically in Ha-RAS-transformed cells [31]. They had reported that flavonoid quercetin drastically reduces the half-life of oncogenic Ras, and Ras protein levels in cell lines expressing oncogenic Ras proteins. Quercetin induces autophagic processes in Ha-RAS-transformed cells. Microtubule-associated protein light chain 3 (LC3) protein is localized in autophagosomes and autolysosomes membranes after processing in quercetin-treated cancer cell lines.

In this report, we also prepared pancreatic cancer peritonitis models using ip injection of AsPC-1 cells. In this model, bloody ascites and multiple tumor nodules were seen at the mesentery after 16 ip days. We administered ip injection of AdCA*tob* to mice bearing pancreatic cancer peritonitis. Tumor growth was suppressed 10 days after ip injections of AdCA*tob* compared to the control group. Our new model of gene therapy for pancreatic cancer by AdCA*tob* in the first week induced significant tumor reduction and complete tumor regression. There was no tumor nodule in the abdomen and no bloody ascites. AdCA*tob* has shown no significant toxic effect on untransformed cells. AdCA*tob* treatment produced significant growth inhibition both *in vivo* and *in vitro*.

Overexpression of wt p53 triggered a short-term cellular response leading to irreversible growth arrest and senescence [32–34]. The commitment to senescence became irreversible with in 48–72 h and no longer required p53 expression. A number of studies utilizing adenoviral or retroviral vectors have evaluated the anti-proliferative and anti-tumorigenic potential of restoration of wild-type p53 in *in vitro* as well as *in vivo* animal models of cancer [35–40]. Nielsen et al. reported that ip injection of AdCMV*wt p53* resulted in reducing the tumor burden of SK-OV-3 ovarian cancer *in vivo* [2]. We also performed ip injections of AdCMV*wt p53* (5×10^7 pfu/day, from day 5 to day 9) after 4 days of ip inoculation of AsPC-1 cells, and tumor growth was suppressed 10 days after ip injections of AdCMV*wt p53* (data not shown). Tumor suppressor gene *p53* includes the regulation of G1-associated cell growth inhibition, maintenance of genomic integrity, control of the apoptotic pathway, and regulation of inhibitors of angiogenesis. Consequently, reconstituting the normal p53 function in tumor cells with defective p53 via introduction of the *wt p53* gene may have therapeutic utility [41,42].

It has been known that the adenovirus vector-mediated gene transfer system has several limitations for *in vivo* application, including transient expression of transferred gene and immunogenic response of the host against adenovirus. As an extension of this study, we are now evaluating the *tob* gene therapeutic potential with a cationic liposomal delivery system to a pancreatic cancer model. Injection of the AdCA*tob* vector provides significant growth inhibition of tumor progression, and our study offers strong support for the gene therapeutic potential of this vector in human pancreatic cancer peritonitis. Continuous progression of these investigations in the future will be necessary for the successful development of a new treatment modality for clinical trials of gene therapy for metastatic pancreatic cancer.

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Chapter 3

BASOPHILIC GRANULOCYTES

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ABSTRACT

Basophilic granulocytes (basophils) are a very small population of peripheral blood leukocytes. Because basophils have high-affinity immunoglobulin E receptors (FcεRI) and secrete chemical mediators that contain histamine, they are thought to be very similar to mast cells. However, research characterizing the function of basophils was slow to proceed and their unique function and importance were not established for a significant period of time. Recently, a series of studies have characterized the role of basophils in anaphylactic shock, chronic allergic reactions, and other human immunological reactions. These studies have shown that basophils are not a supplementary cell type but key players in very serious immune reactions. In this chapter we introduce the recently discovered characteristics of basophils. In addition, we consider how these aspects are clinically important and are connected with new cellular or molecular treatments for allergic reactions.

INTRODUCTION

Basophilic granulocytes (basophils) are a small population of peripheral blood leukocytes that were first described in 1879 by Ehrlich[1]. These cells contain unique cytoplasmic granules that stain with basophilic dyes, such as toluidine blue. The percentage of basophils in the peripheral blood is low (<1%), and they share physicochemical properties with other blood cells. Like mast cells, basophils possess high-affinity immunoglobulin (Ig) E receptors (FcεRI) that are cross-linked when the receptor bound IgE is engaged with the corresponding antigen ("allergen"). Receptor cross-linking results in the release of a number of mediators, which contain some elements that are common to both cell types. Based on their similarity to mast cells, basophils have been thought to play a minor and possibly redundant role as "circulating mast cells"[2, 3].

Not so long ago, some investigators thought that mice entirely lacked basophils because they could not be detected by normal hematologic staining (eg.Wright-Giemsa). In 1981, Urbina et al. showed that mouse basophils have a distinct morphology that is different from other species, which may have contributed to their inability to be detected in routine skin preparations[4]. Furthermore, the ultrastructural features of mouse basophils have been well defined[5], and many studies have been conducted on this unique cell population[6, 7].

Phenotype, Development, and Activation of Basophils

The early stages of basophil maturation and their relationship to other cell lineages are not well understood[2]. Basophils express a variety of cytokine receptors (IL-1RII (CD121b), IL-2Rα (CD25), IL-3Rα (CD123), IL-4Rα (CD124), IL-8R (CD128), GM-CSFRα (CD116), IFNRγ (CD119)), chemokine receptors [CCR1, CCR2, CCR3, CCR5 (CD195), CXCR1 (IL-8Rα), CXCR2 (IL-8Rβ), CXCR4 (CD170), CRTH2], complement receptors [CD11b (iC3bR), CD11c (C3biR), CD21 (C3dR), CD35 (C3bR, C4bR), CD45 (C3bR, C4bR), CD55 (C4b/2aR, C3b/BbR), CD59 (C5b-8R, C5b-9R)], homing receptors and related molecules [CD15s, CD62L, CD162, CD11a (LFA-1), CD18, CD29, CD44 (Pgp-1), CD49a (VLA-1), CD49d (VLA-4)], prostaglandin receptors, and Ig Fc receptors [CDw32 (FcγRIIA and B), FcεRI, FcγRIII[8-10].

Previous reports have suggested that basophils evolved from eosinophil/basophil progenitors, and this hypothesis is supported by the presence

of granulocytes with hybrid eosinophil/basophil phenotypes in patients with chronic or acute myelogenous leukemia and in cell culture[11-13]. On the other hand, the possibility that mast cells and basophils share a common lineage arises from the observation that basophils with phenotypic features that are characteristic of mast cells can be found in patients with asthma, allergies, or allergic drug reactions[14]. Therefore, the current predominant model that mast cells and basophils originate from separate lineages is still debated and may have to be revised[2].

Our understanding of basophils has been advanced by the development of basophil-specific monoclonal antibodies, Bsp-1, 2D7, BB1 and 212H6[10, 15-17]. In addition, the 97A6 monoclonal antibody has been described as an antibody specific for mature mast cells, basophils, and their progenitors[18]. 97A6 does not react with any other hematopoietic or nonhematopoietic cell types. The epitope recognized by 97A6 may therefore be associated with the commitment of the CD34 precursor to a mast cell or basophil lineage that is distinct from other lineages[2].

Among the many cytokines that stimulate basophils, IL-3 is thought to be the main growth and differentiation factor for basophils[19, 20]. SCF together with IL-3 expand the progenitor pool of most hematopoietic cell types in the bone marrow, including mast cells and basophils[20].

As with mast cells, basophils express complete and functional FcεRI receptors, and cross-linking of these receptors leads to basophil activation, granule exocytosis and mediator release[21]. C3a and C5a can also activate basophils through the C3aR and C5aR complement receptors, respectively. Activation through any of these receptors leads to histamine release, eicosanoid synthesis, and IL-4 and IL-13 gene expression. In addition, basophil activation may be associated with increased CD18 and CD63 expression and decreased Leu-8 (CD62L) expression[8].

Basophils and Anaphylaxis

Anaphylaxis is an immune reaction that is induced upon exposure to food, wasp toxins, and allergens, such as medicine and latex, and can lead to a severe generalized allergy[22, 23]. Because anaphylaxis is often associated with rapid skin urticaria, decreased blood pressure, dyspnea, consciousness disorder, etc., this condition is dangerous and sometimes fatal. Therefore, anaphylaxis is a pathosis that concerns medical professionals and both rapid diagnosis and treatment are necessary. Previous work has shown that both mast cells and

basophils perform important functions in anaphylaxis however, more recently it became clear that mast cells and basophils have quite different mechanisms and chemical mediators (Figure 1)[23].

(1) Classical pathway of anaphylaxis by mast cells and IgE

Mast cells are prevalent in the skin, mucous membrane, and the circumvascular, and they become sensitized to an allergen when allergen-IgE complexes bind the high-affinity IgE receptor FcεRI expressed on their cell surface. When re-exposed to the same allergen, the allergen cross-links the IgE/FcεRI complex on the surface of the mast cell, and an activation signal is transmitted that induces mast cell degranulation and the extracellular release of secretory granules that contain histamine and other chemical mediators[24, 25]. As a result, vascular permeability increases, bronchus smooth muscles contract, etc., leading to the characteristic anaphylactic symptoms such as rapid hypotension and dyspnea[26].

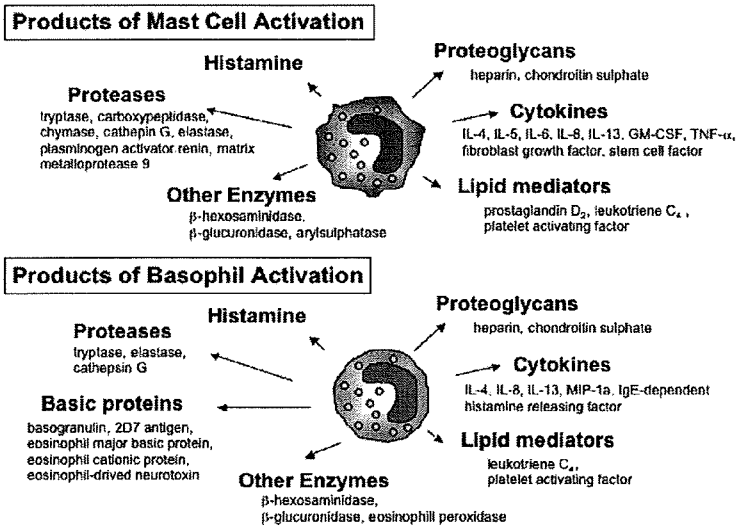

Figure 1. A. Mast cells and their activation products. B. Basophils and their activation products. Figure courtesy of Dr. A.F. Walls (Reference 23). IL, Interleukin; GM-CSF, Granulocyte-macrophage colony stimulating factor; TNF, Tumor necrosis factor; MIP, Macrophage inflammatory protein.

It was recently shown that anaphylactic pathosis cannot be completely attributed to the classical pathway based on analyses of a mouse model of the 'classical' pathway, which consists of IgE, FcεRI, mast cells, and histamine. For example, mast cell-deficient, IgE-deficient, or FcεRI-deficient mice still exhibited generalized anaphylaxis when they were sensitized to an allergen and then re-exposed to the same allergen[27-31]. These findings strongly suggest that there is another anaphylactic route that is distinct from the classical pathway (Figure 2)[32].

(2) A new anaphylaxis pathway that involves basophils and IgG

Mice lacking the FcRγ chain common to FcεRI, which is the IgE receptor, and FcγRI/III, which is the IgG receptor, do not experience anaphylactic reactions, suggesting that there is an IgG-mediated anaphylactic response in addition to an IgE-mediated reaction. After these mice were given antigen-specific IgG_1 monoclonal antibodies and then challenged with intravenous allergen, systemic anaphylaxis with tachycardia was induced[29]. Furthermore, it was ascertained that IgG_1-mediated anaphylaxis is induced in mast cell-deficient mice, which strongly suggest that a type of IgG_1-mediated, mast cell-independent anaphylaxis exists[27, 29]. Macrophages were considered a potential cell type that is responsible for IgG_1-mediated anaphylaxis[31], but there has been no conclusive proof establishing their role in this response. Because the high-affinity IgE receptor, FcεRI, is expressed on both mast cells and basophils in mice, it is conceivable that basophils are also are responsible for IgE-mediated anaphylaxis[33].

Tsujimura et al. developed a mouse monoclonal antibody that can deplete basophils in vivo. Using this antibody, they showed that basophils were dispensable for IgE-mediated anaphylaxis, while mast cells were critical for the "classical" anaphylactic pathway mediated by IgE and histamine. They injected allergen-specific IgE into basophil-depleted mice, and then challenged these mice with allergen. The anaphylactic response occurred in the basophil-depleted mouse, while mast cell-deficient mice exhibited no anaphylactic responses[34].

They also developed a penicillin G–specific mouse monoclonal IgG_1 antibody, and injected this IgG1 antibody into basophil-depleted mice. While an anaphylactic reaction occurred in control mice that were injected with penicillin G combined with bovine serum albumin, basophil-depleted mice did not have an anaphylactic response. Interestingly, this IgG-dependent anaphylactic response was more severe than the IgE-dependent response. Furthermore, these responses occurred in both normal and mast cell-deficient mice. Collectively, these

experiments provided seminal evidence that basophils play an important role in IgG-dependent anaphylaxis [34]. The allergen-specific IgG_1 and allergen complexes were found mainly on the surface of basophils, but also on macrophages, neutrophils, and natural killer cells by flow cytometry analysis. However, if these cell types except basophils were depleted from the mice prior to injecting the antibody and allergen, there was still an IgG-dependent anaphylactic response[34]. These results strongly indicate that basophils are a key component of IgG-dependent anaphylaxis.

(3) Platelet-activating factor and basophil-mediated anaphylaxis

Unlike IgG-dependent anaphylaxis, IgE-dependent anaphylaxis in mice is prevented by pre-treating with an anti-histamine drug. Based on these findings, the intervention of chemical mediators other than histamine likely contributes to IgG-dependent anaphylaxis[34]. Platelet-activating factor (PAF) is also known to act on the vascular endothelium and facilitate its permeability. When mice were injected with a PAF antagonist, IgG-dependent, but not IgE-dependent, anaphylaxis was almost completely inhibited. The stimulation by allergen and allergen-specific IgG_1 caused a significant elevation in PAF production, only in basophils. Furthermore, when conditioned medium from basophils that were stimulated with immune complexes was added to human vascular endothelial cells (normal human umbilical vein endothelium cells: HUVEC), their intercellular space expanded as an indication of vascular permeability; furthermore, this phenotype was inhibited with a PAF antagonist. From these results, it became clear that basophils quickly combine the immune complexes that are formed in the blood through the IgG receptor, which induces the production and release of factors such as PAF that ultimately lead to systemic anaphylaxis[34]. Although basophils are a minor population of cells in the blood and occupy only 0.5% of peripheral leukocytes, when they are activated by immune complexes they induce strong systemic anaphylaxis by secreting PAF, which induces vascular permeability 1,000-10,000 times higher than histamine.

(4) The role of basophils in human anaphylaxis

The experiments described above analyzed passive anaphylaxis, which is induced by challenging with an allergen after administering allergen-specific IgE or IgG. To examine anaphylaxis reactions that are more similar to human allergic conditions, Tsujimura et al. examined the role of basophils in active anaphylaxis by immunizing mice with an allergen two weeks prior to an intravenous injection of the allergen. This active anaphylaxis is more serious than passive anaphylaxis and caused fatal anaphylactic shock not only in normal mice but also in mast cell-

deficient mice. However, death from anaphylactic shock was prevented in mast cell-deficient mice when they were pretreated with the basophil-depleting antibody. This finding provided clear evidence that basophils have a decisive role in active anaphylaxis. Interestingly, fatal anaphylactic shock was not prevented in basophil-depleted normal mice that have mast cells. Therefore, these results indicate that both the classical pathway caused by mast cells and the new pathway caused by basophils equally contribute to anaphylactic shock[34].

Although it is not clear whether these results in mice apply to humans, there are many human reports that suggest there is an alternative pathway in addition to the classical pathway. There are reports of clinical anaphylactic cases, especially due to drug allergies, where allergen-specific IgE was not detected[35], mast cell tryptase was not elevated[36], and allergen-specific IgG antibody was elevated[37-39]. Recently, Vadas P. et al. showed that serum PAF levels were directly correlated and serum PAF acetylhydrolase activity was inversely correlated with the severity of anaphylaxis, and that the failure of PAF acetylhydrolase to inactivate PAF may contribute to the severity of anaphylaxis[40].

Based on analyses in mice, the new basophil-mediated anaphylaxis pathway is thought to require more allergens and antibodies than the classical pathway[34]. Therefore, when large amounts of soluble material are introduced into the body, such as antibody therapy, which has received recent attention as a molecular therapy, both the classical pathway and the new pathway may contribute to intense anaphylactic shock. Moreover, when we use peptide-specific immune therapy for autoimmune diseases or hyposensitization treatment for allergic diseases to induce immunologic tolerance, there is a danger that these therapies will lead to IgG-mediated anaphylaxis. Therefore, high-risk patients should be monitored not only for IgE levels but also for allergen-specific IgG, basophil function and/or serum PAF levels.

Roles of Basophils in Chronic Allergic Reactions

Basophils are often recruited to the site of allergic inflammation. However, for a long period of time, there was no definitive evidence that basophils were crucially involved in the pathogenesis of chronic allergic disorders. Mukai et al. showed that basophils are responsible for the development of IgE-mediated chronic allergic inflammation independently of T cells and mast cells[41]. Using a chronic cutaneous allergy mouse model they showed that basophils act as an initiator cell rather than an effector cell in chronic allergic inflammation.

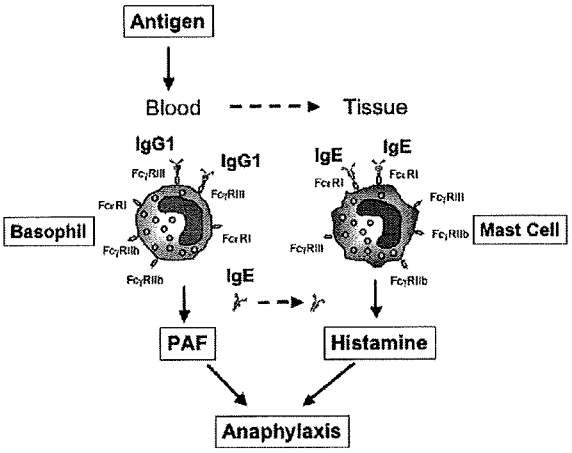

Figure 2. Schematic of the two anaphylaxis pathways. The classical pathway involving mast cells is initiated when antigen-IgE immune complexes bind FcεRI on the cell surface. The anaphylactic reaction is caused by the release of histamine from mast cells. The new pathway involving basophils begins when antigen-IgG_1 immune complexes bind to FcγRIII surface receptors, and platelet-activating factor (PAF) is released from basophils.

(1) Roles of IgE in the chronic allergic reaction

Matsuoka et al. established transgenic mice that carry genes encoding the heavy and light chains of hapten trinitrophenol (TNP)-specific IgE to generate a model system that would help elucidate both the pathological roles of IgE in the acute and chronic phases of allergic inflammation and the immunobiological roles in vivo. These mice produced high titers of TNP-specific IgE and their mast cells were heavily loaded with IgE[42]. According to their colleague's report, the immediate-type allergic ear swelling response of the biphasic response appeared after the ear of the mouse was intracutaneously challenged with the homologous TNP-binding ovalbumin[43]. Furthermore, on the second day of the antigenic challenge the ear began to swell again and more than doubled in skin thickness on the fourth day. Pathologic histology was used to show the detailed aspects of chronic allergic inflammation; for example, there was a robust infiltration of cells including basophils, and cornification was recognized as hyperplasia of the epidermis. The third phase of ear swelling was shown to be antigen-specific and IgE-dependent and it could be induced when antigen-specific IgE was given to normal mice (passive sensitization) prior to the antigen challenge. This

phenomenon was seen both in transgenic and wild-type mice. It was proven that IgE contributed not only to the immediate-type allergic response but also to the chronic allergic inflammatory response[41].

(2) The role of basophils in the IgE-dependent chronic allergic inflammatory response

When mast cell-deficient mice were challenged with the antigen after passive sensitization of IgE, the first and second phases of ear swelling, which are the immediate-type allergy response, were not observed, while the third-phase of ear swelling was observed both in these mice and in normal mice[41]. Based on this phenomenon, it is thought that mast cells are dispensable for the IgE-dependent chronic allergic response and that the immediate-type allergic response is required to induce the chronic response. Cyclosporine A almost completely inhibited the third phase of ear swelling and cellular infiltration, whereas an anti-histamine, cyproheptadine, did not have any significant effects on the third phase of the reaction. Given the delayed time of ear swelling, T cells were thought to contribute to this response. However, the third phase of ear swelling was also observed in T cell-deficient mice and T cells were not essential for the initiation of IgE-dependent chronic allergic inflammation[43].

Neither immediate ear swelling nor the third phase of ear swelling was observed in mice deficient for FcεRI, which is a high-affinity IgE receptor. This result indicates that the third phase of ear swelling requires a cell type that expresses FcεRI on its surface. To identify this cell type, Mukai et al. transferred various cells from normal mice into FcεRI-deficient mice and studied whether the third phase of ear swelling was restored. When FcεRI-expressing basophils that also express the natural killer cell marker DX5 (CD49b) were transferred into FcεRI-deficient mice, the third phase of ear swelling was restored. These findings indicate that this novel mechanism that leads to the development of chronic allergic inflammation is induced by basophils through the interaction of antigen, IgE, and FcεRI[41].

(3) Basophils as potential therapeutic targets for chronic allergic inflammation

When Mukai et al. examined the cells that infiltrated into the skin during the third phase of ear swelling skin, basophils comprised only 1-2% of the infiltrate and most of the cells were eosinophils and neutrophils. Therefore, they wanted to determine how this minor basophil population could cause chronic allergic inflammation[41].

Using the basophil-depleting monoclonal antibody established by their colleagues, they confirmed that this antibody could markedly decrease the number of basophils and further showed that mice pretreated with this antibody did not exhibit the third phase of ear swelling[44, 45]. This result proves that basophils are responsible for IgE-dependent chronic allergic inflammation. Furthermore, when this basophil-depleting antibody was given 2 or 3 days after allergen injection and the third-phase ear swelling had already occurred, the ear swelling and inflammation were inhibited and eosinophil and neutrophil infiltration was decreased markedly[44]. This result suggests that basophils function more as an initiator cell than as an effector cell and indicates the possibility that chronic allergic inflammation could be treated by targeting basophils. Once activated by allergen-mediated cross-linking of IgE/FcεRI, basophils secrete humoral factors such as a cytokines and chemokines that may directly or indirectly contribute to the infiltration of eosinophils and neutrophils.

The Role of Basophils in the Control of T Cell Differentiation

(1) T cell development and IL-4

CD4 positive T cells are functionally divided into four types; Th1, Th2, and Th17 are helper T cells[46-49], while Tregs are regulatory T cells[50]. Naive T cells differentiate into these functional T cells in response to different cytokines. IL-4 plays a crucial role in the development of Th2-type immune responses and the regulation of immunoglobulin isotype switching to IgE[51-53]. The IL-4-producing cell that produces sufficient levels of IL-4 to differentiate naive cells into Th2 cells in the lymph node is still unknown. Because Th2 cells produce variable amounts of Il-4, T cells themselves may be the predominant IL-4-producing cell that stimulates naive T cells to differentiate into Th2 cells[51, 54]. On the other hand, dendritic cells (DCs) and macrophages are required for CD4+ T cells to develop into Th1 cells. DCs are generally divided into three types; DC1 cells express the highest levels of MHC class I, class II, CD40, B7.1 and B7.2 compared to DC0 and DC2 cells. In terms of IL-12 production, DC1 cells have enhanced production, while DC2 cells produce lower levels than DC0 cells. Both DC0 and DC1 supported the differentiation of IFNγ-producing Th1 cells, but not IL-4-producing Th2 cells from TCR-transgenic naïve mouse Th cells. However, DC2 cells selectively enhanced the differentiation of IL-4-producing Th2 cells[55].

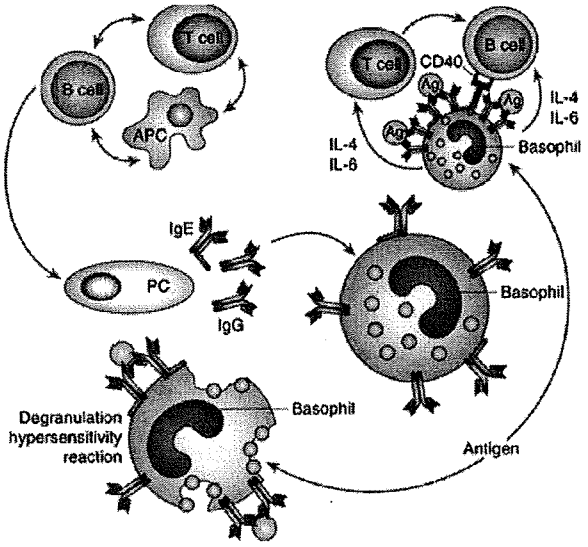

Figure 3. Basophils are mediators of the immune response. Upper left: Classical primary response by CD4+ T cells, B cells, and APCs, Ag-presenting cell (APC); PC, plasma cell. After IgG and IgE are produced by plasma cells (PC), basophils bind these circulating Abs. Upper right: A second presentation with Ag results in Ag-loaded basophils that begin producing the cytokines IL-4 and IL-6. Ag presentation and cytokine production by basophils enhance the secondary humoral immune response (upper right). The hypersensitivity reaction is caused by basophil degranulation and the release of lipid mediators (lower left). (Reference 64)

(2) The roles of basophils in Th2 cell development

Based on previous studies, basophils along with mast cells were thought to be cell types that produce chemical mediators such as the histamine and leukotoluene and cause allergic reactions. However, recently, basophils have received much attention because they have been shown to produce massive amounts of IL-4 when activated[56-59]. A basophil produces ten-fold more IL-4 than a Th2 cell. When naïve T-cells were cultivated with activated basophils in vitro, the naïve T cells differentiated into Th2 cells as a direct result of the IL-4 that was secreted by basophils[60, 61]. In addition, it has been established that parasitic infected mice have an abundance of differentiated Th2 cells. It was also reported that basophils produced IL-4 under such conditions[58, 59]. However, it is still unknown whether the IL-4 produced by basophils initiates Th2 cell differentiation or

maintains the Th2 cell dominant status. Furthermore, it was also unknown whether basophils interact with the naïve T cells in vivo. Naïve T cells differentiate into Th2 cells in the peripheral lymph node, and it is thought that basophils, unlike lymphocytes, circulate in the peripheral blood and never enter the peripheral lymph nodes. However, Sokol et al. recently elucidated a novel role of basophils in immunized mice[62]. They noted that many allergens have protease activity; therefore, they injected the protease papain into mice and then examined the secretion of IL-4 and other Th2-inducing cytokines. They showed that basophils transiently entered the regional lymph nodes soon after papain administration and that basophils secreted IL-4 after direct stimulation with papain. These results suggest that basophils also have important roles in the initial stages of immunization.

Roles of Basophils in Immunological Memory Responses

The cellular basis of immunological memory remains controversial. The classical primary immune response by CD4+ T cells, B cells, and antigen–presenting cells (APC) is initiated when antigen is presented to the host. Following IgG and IgE production by plasma cells (PC), basophils bind circulating antibody of either class (Figure 3). Denzel et al. showed that basophils bound large amounts of intact antigens on their surface and were the main source of IL-4 and IL-6 in the spleen and bone marrow after restimulation with a soluble antigen[63]. They also showed that basophil depletion resulted in a much lower humoral memory response and greater susceptibility of immunized mice to *Streptococcus pneumoniae*-induced sepsis. Adoptive transfer of antigen-reactive basophils significantly increased specific antibody production, and activated basophils, together with CD4+ T cells, profoundly enhanced B cell proliferation and immunoglobulin production. These basophil-dependent effects on B cells required IL-4 and IL-6 and increased the capacity of CD4+ T cells to provide B cell help (Figure 3. upper right). The circumstances that differentiate this participatory role of basophils from its traditional mast cell-like reaction, consisting of full degranulation, lipid mediator release, and cytokine release and subsequent anaphylactic reaction, remain unknown (Figure 3)[64].

Although basophils are not an abundant cell type and have been previously overlooked, they have recently emerged as highly important immune cells. Because anaphylactic shock and other serious reactions that involve basophils are closely related and can cause serious conditions, it is possible that basophils are maintained at low numbers to avoid these conditions. However, because they are usually low in number, they may be easy to target for treatment. Because brilliant