5. Retroviral vector-producing mesenchymal stem cells for tumor tracking and therapeutic gene amplification in suicide cancer gene therapy

MSCs are known to have a tendency to accumulate at the site of tumors, and therefore can be utilized as a platform for targeted delivery of anti-cancer agents [21–23]. The MSC-based targeted cancer gene therapy can enhance the therapeutic efficacy, because MSCs are considered to reach tumors including metastatic lesions and to deliver therapeutic molecules in a concentrated fashion. This targeted therapy can also reduce systemic adverse side effects, because the anti-cancer agents act locally at the site of tumors without elevating their systemic concentrations. We developed genetically-modified MSCs that produce retroviral vectors encoding HSV*tk*, aiming at augmenting therapeutic efficacy of systemic suicide cancer gene therapy (Fig. 3). The tumor tropism and anti-tumor effects of vector-producing MSCs (VP-MSCs) were examined by intravascular injection in tumor-bearing nude mice. MSCs isolated from the bone marrow of SD rats were transfected with plasmid DNA expressing luciferase alone (=non-VP-MSCs) or whole retroviral vector components (LTR-Luc or LTR-HSV*tk* with Gag-pol and VSV-G) (=VP-MSCs) by nucleofection. To assess tumor tropism of MSCs, nude mice were subcutaneously inoculated with 9 L rat glioma cells or Rat-1 fibroblasts, and were subsequently injected with luciferase-expressing MSCs through the left ventricular cavity. The transgene expression was periodically traced by using an *in vivo* imaging system. As a result, the transgene expression accumulated at the site of subcutaneous 9 L tumors, but undetectable at the site of Rat-1 fibroblasts. In addition, the injection of luciferase-expressing VP-MSCs caused much stronger signal of bioluminescence at the site of 9 L tumors compared with luciferease-expressing non-VP-MSCs. Immunostaining study showed that luciferase-positive cells (injected MSCs and transduced glioma cells) were detected at the periphery of tumors. To evaluate the therapeutic efficacy, tumor-bearing nude mice were treated with non-VP-MSCs or VP-MSCs combined with HSV*tk*/GCV system and then the size of subcutaneous tumors was periodically measured. In this model experiments, tumor growth was more efficiently suppressed by injecting VP-MSCs compared with non-VP-MSCs (Uchibori R, et al.: manuscript in preparation). This study suggests the effectiveness of VP-MSCs in suicide cancer gene therapy. The therapeutic benefit of this strategy should be further examined in orthotopic and metastatic tumor models.

6. Site-specific insertion of a therapeutic gene into the AAVS1 locus (19q13.4) in human mesenchymal stem cells by using adeno-associated virus integration machinery

Hematopoietic stem cells, ES cells, and MSCs are attractive targets for gene therapy and regenerative medicine, since they replicate themselves and differentiate into various cell lineages. To introduce genes in these stem cells, it is especially important to utilize a system that results in a minimal risk of insertional mutagenesis. To date, only one animal virus, the adeno-associated virus (AAV), is able to integrate into a defined site in human chromosome, AAVS1 (19q13.4), which is mediated by the activity of specific replicase/integrase protein, Rep. The Rep78 or Rep68 protein recognizes the GAGC motif on the viral inverted terminal repeat (ITR) sequence and a similar motif in AAVS1, leading to the site-specific integration of the AAV genome.

We and others have reported that a plasmid transfection system utilizing AAV derived components, the *rep* gene and ITR, could integrate the gene of interest preferentially into AAVS1 in epithelial or adherent cells (e.g., 293, HeLa, Huh-7 cells) [24–26]. Our system uses two plasmids, one harboring the transgene cassette flanked by the ITR sequences, and the other for *rep* expression, allowing only plasmid DNA harboring the ITR to integrate into the AAVS1 locus. In addition, this system can deliver DNA segments larger than the 4.5-kb packaging limit of AAV. As a first step toward establishing a method capable of integrating therapeutic DNA into the AAVS1 locus in MSCs, we tested this strategy in KM-102 cells, a cell line derived from human marrow stromal cells. KM-102 cells were co-transfected with a bicistronic plasmid containing a humanized GFP gene and a blasticidin S resistance gene (*bsr*) between the ITRs and a Rep68 plasmid. After transfection, single cell clones were grown in the presence of

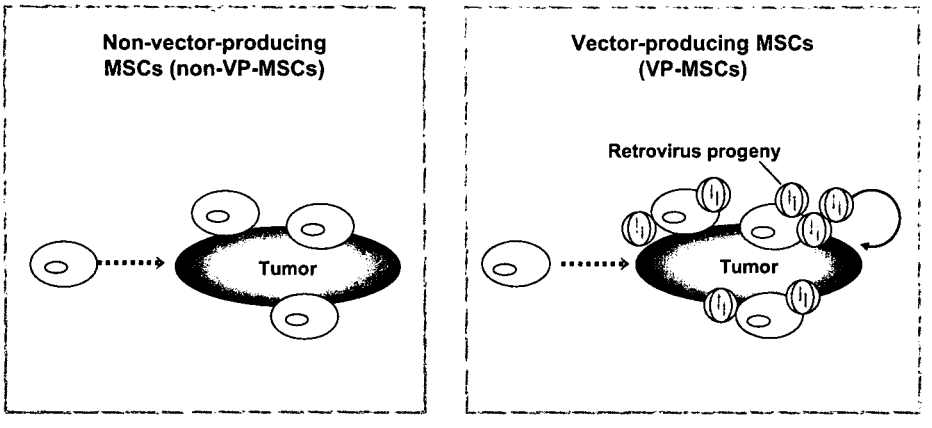

Fig. 3. Development of vector-producing tumor-tracking MSCs to augment suicide cancer gene therapy.

blasticidin S. Southern blot analysis of their genomic DNA revealed that three out of eight blasticidin S resistant clones showed site-specific integration of transgene into the AAVS1 site and that these clones had the GFP gene only at AAVS1. These results indicated that foreign DNA linked with ITR sequence could be targeted specifically into AAVS1 in KM-102 cells.

It is reported that the genome of myosin binding subunit 85 (MBS85) overlaps with the AAVS1 site [27]. To identify the junction between the transgene plasmid and the AAVS1 site, PCR was conducted using a transgene- and an AAVS1-specific primers. In two of the three clones the integration site was identified. In one clone the GFP gene was inserted at the first intron of MBS85 gene. The other clone had insertion of the GFP gene upstream of the first exon. Quantification of mRNA for MBS85 by real time PCR showed that the mRNA level decreased in these two KM-102 clones. The MBS85 is involved in the assembly of actin cytoskeleton. Although the outcome of allelic disruption of the MBS85 genome should be carefully evaluated, the system for AAVS1-specific integration of therapeutic DNA using AAV integration machinery is particularly valuable for *ex vivo* gene therapy applications for stem cells, such as ES cells and MSCs. For additional readings on the use of bone marrow cells for the treatment of autoimmunity, the reader is referred to companion papers published herein in this special issue of the Journal of Autoimmunity [28–38].

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Vector-producing tumor-tracking multipotent mesenchymal stromal cells for suicide cancer gene therapy

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1. ABSTRACT

Suicide cancer gene therapy with retroviral vector-producing cells was in the way of an adjuvant to the surgical resection of recurrent glioblastoma, although any benefit appeared to be marginal. It is likely that this therapeutic approach may have better outcomes if the vectors and transgenes are delivered more efficiently to the tumor cells. We have shown previously that tumor cells engineered by adenovirus-retrovirus hybrid vectors to produce retroviral progeny destroy satellite tumor cells. Whether the systemic delivery of vector-producing cells can effectively treat aggressive tumors remains to be determined. Effective retroviral vector delivery vehicles may be multipotent mesenchymal stromal cells (MSCs), which have been shown to home to tumor cells *in vivo* and deliver cancer-killing gene or immune products with minimal host rejection. Therefore, it may be possible to transduce tumors with recombinant progeny vectors delivered by MSCs. This may be particularly suitable for treating diffuse cancers like glioblastoma multiforme. While this strategy remains to be tested in various orthotopic or metastatic tumor models, it has the potential to greatly improve the outcome of suicide gene therapy.

2. SUICIDE CANCER GENE THERAPY USING VECTOR-PRODUCING CELLS

A gene therapy against glioma has been developed in which a cell line that continuously secretes a retroviral vector is implanted into brain tumors (1). The vector, which expresses the herpes simplex thymidine kinase (HSV-*tk*) transgene, "infects" the local tumor cells, which then become susceptible to tumoricidal metabolites generated by HSV-*tk*-mediated activation of the prodrug ganciclovir (GCV). The therapeutic value of this technique appeared to be enhanced by a bystander effect wherein the transduced tumor cells communicate the apoptosis signal to neighboring cells. However, while this cancer gene therapy system was shown to have some clinical benefit, its efficacy was limited due to the poor efficiency of gene transfer (2). To improve the therapeutic potential of this system, it is necessary to enhance (a) the efficiency of therapeutic gene delivery *in vivo* and (b) the stability of the vector-producing cells.

With regard to therapeutic gene delivery *in vivo*, we have previously described a hybrid vector system where adenoviral vectors are used to deliver retroviral vector andpackaging proteins into cells (3). This system benefits

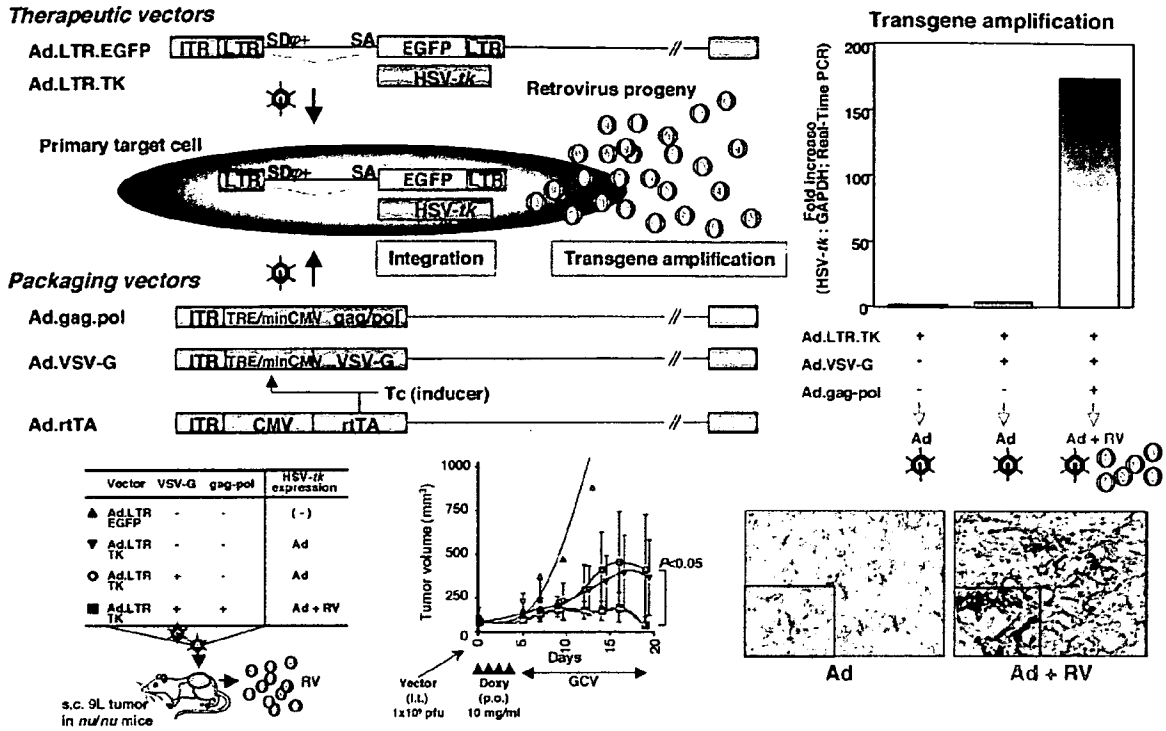

Figure 1. Two adeno-retroviral hybrid vectors containing a retroviral vector genome (Ad.LTR.EGFP or Ad.LTR.TK) were constructed. Co-transduction of rat 9L glioma cells with an adeno-retroviral hybrid vector together with vectors expressing retrovirus packaging proteins (Ad.gag.pol, Ad.VSV-G) as well as an inducer (Ad.rtTA) increased the transduction efficiency. Injection of established subcutaneous 9L tumors on athymic mice with a combination of AVC2.GCTK and packaging vectors followed by GCV treatment resulted in complete regression by 50% of the tumors at day 22, while no tumor regression was observed in control animals. Furthermore, the relative copy number of the HSV-tk gene in tumors treated with the adeno-retroviral vectors was significantly higher than in control tumors. In situ hybridization analysis also suggested dispersion of the HSV-tk product across a wider area of the tumor than in control tumors, which indicates the spread of the in situ-generated retroviruses.

from the efficient gene transfer characteristics of adenoviral vectors as well as the stable and long-term gene expression that is typical of retroviral vectors. We have shown that direct transduction of primary target tumor cells with hybrid adeno-retroviral vectors results in their transient production of recombinant retrovirus particles that then subsequently transduce neighboring tumor cells (3). Moreover, when we transduced established subcutaneous 9L tumors on athymic mice *in situ* with adenovirus vectors that express transcomplementing genes encoding retroviral proteins and retroviral vector RNAs, upon GCV treatment, 50% of the tumors showed complete regression at day 22, while no tumor regression was observed in control animals (Figure 1). This strategy can now be developed further by using cells with tumor-tracking properties as the vector-producing cells, thereby targeting the therapeutic gene to the tumor cells *in vivo*.

3. MULTIPOTENT MESENCHYMAL STROMAL CELLS (MSCs) AS A PLATFORM FOR VECTOR PRODUCTION *IN SITU*

We propose here an improved *in situ* vector production strategy where cells bearing tumor-tracking properties efficiently produce retrovirus- or other virus-based progeny vectors (Figure 2). Candidate tumor-tracking vector-producing cells are adult stem cells. In particular, the fibroblast-like plastic-adherent cells isolated from bone marrow and other sources that are now widely known as mesenchymal stem cells or multipotent mesenchymal stromal cells (MSCs) (4), may be useful as they have been shown to have tumor-seeking properties (5). While the mechanism that induces MSCs to preferentially engraft themselves in tumors remains poorly undertsood, this phenomenon may be mediated by the cytokines released by the tumor or inflammatory tissue. These include hepatocyte growth factor (HGF) (6), vascular endothelial cell growth factor (VEGF) (7), transforming growth factor (TGF) (7), fibroblast growth factor (FGF) (6), platelet-derived growth factor (PDGF) (8), monocyte chemoattractant protein-1 (MCP-1) (9), and IL-8 (9). Moreover, chemokine C-X-C motif receptor 4 (CXCR4), which is present on the surface of an MSC subset, is known to mediate not only the specific migration of MSCs to bone marrow (10), it also governs the migration and homing of a variety of cell types in the developing brain, including neuronal and glial precursors. The only known chemokine that binds with CXCR4 is stromal-cell derived factor-1 (SDF-1). While CXCR4 itself is a major chemokinereceptor on glioma cells and promotes their

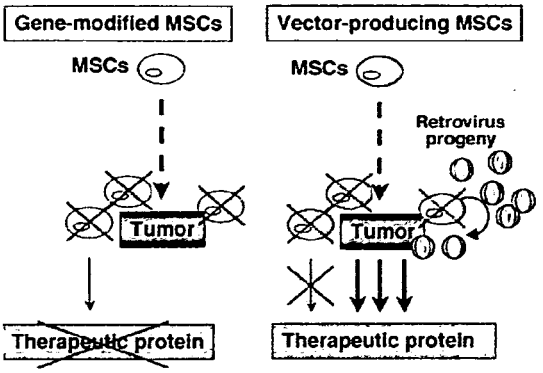

Figure 2. Left panel; Gene-modified MSCs. Although gene-modified MSCs have tumor-seeking properties, the local expression of the therapeutic protein is dependent on the continuing presence of the MSCs. Right panel; Vector-producing MSCs. The retrovirus progeny produced by the MSCs can transduce tumor cells *in situ*, which extends the expression of the therapeutic protein, even when the MSCs die off.

survival (11), high-grade gliomas have recently been found to secrete significant levels of SDF-1 (12). SDF-1 alpha stimulates human glioblastoma cell growth by activating both extracellular signal-regulated kinases 1/2 and Akt. Therefore, CXCR4 expression by MSCs may help them to home to gliomas. When MSCs are infused in mice, they are rapidly and efficiently arrested in the microvasculature (13). Furthermore, these cells are not immunogenic and escape recognition by alloreactive T cells and natural killer cells (14). It also appears that the engraftment of MSCs into the tumor helps them to maintain their stem cell properties *in vivo*. Thus, it appears that MSCs may efficiently engraft human gliomas after intravascular or local delivery and can be used as *in situ* therapeutic vector producers.

Other candidate tumor-tracking cells that may be used as vector-producing cells include endothelial progenitor cells (EPCs). EPCs have been isolated from peripheral blood CD34, Flk-1, or AC133 antigen-positive cells, which are believed to include a hematopoietic stem cell population, and have been shown to incorporate into neovascularization foci (15). Moreover, VEGF promotes adult vasculogenesis by enhancing EPC recruitment and vessel formation at sites of tumor neovascularization (16). Magnetic resonance imaging (MRI) of magnetically labeled endothelial progenitor cells also demonstrated that they traffick to sites of tumor angiogenesis (17).

4. GENETIC MANIPULATION OF MSCs TO FACILITATE THEIR PRODUCTION OF PROGENY VECTORS

MSCs exhibit senescence-associated growth arrest and phenotypic changes during long-term *in vitro* culture. However, overexpression of human telomerase reverse transcriptase (hTERT) in MSCs reconstitutes their telomerase activity and extends their life span (18). Telomerization of MSCs by hTERT overexpression also maintains the stem cell phenotype of MSCs and thus may be useful for generating the numbers of stable MSCs needed for cell differentiation studies and tissue engineering protocols.

To produce therapeutic vectors, the MSCs must be efficiently transduced with viral components. Virus-based transduction techniques have been shown to achieve high gene transduction and transgene expression in many cellular models, and attempts have been made to transduce MSCs with various virus-based vectors such as oncogenic retrovirus- or lentivirus-based vectors. However, the use of integrating viral vectors has several disadvantages, particularly with regard to their safety risks. Many non-viral methods also have limited utility as they are rather inefficient with most primary cells. However, nucleofection, which is a non-viral electroporation-based gene transfer technique, has been shown to be an efficient non-viral transfection technique for MSCs, which then may be used as cellular vehicles for the delivery of biological agents (19). Thus, the Nucleofector technology may be promising as an alternative tool for efficiently transfecting MSCs so that they produce progeny virus.

5. FUTURE DIRECTIONS

Here we propose that current suicide cancer gene therapy strategies may be improved by using vector-producing tumor-tracking MSCs. This strategy is likely to generate *in situ* the vector numbers needed for the killing of solid tumors. We also showed that it may be feasible to produce large-scale preparations of vector-producing cells by transient transduction of MSCs by hybrid adenovirus-based vector infection. It has been shown that the hybrid adenovirus-based vectors that express retroviral proteins can efficiently transduce cells, which then produce progeny vectors (3). However, an impediment for this aim is that MSCs lack the Coxsackie adenovirus receptor (CAR) (20). To overcome this problem, it may be necessary to use a chimeric Ad35 fiber-containing Ad5 vector (21) or a fiber-modified Ad5 vector bearing an RGD-motif peptide in the HI loop of the fiber knob domain (22). Alternatively, it may be possible to use an adaptor molecule that bridges the gap between the viruses and MSCs. Supporting the latter possibility is that we have previously developed a CAR-SCF fusion protein that improves the transduction efficiency of the adenovirus vector with c-kit positive cells (23). Similar CAR-ligand adaptor molecules may be useful for enhancing MSC transduction with the adenovirus vector.

To improve the tumor-targeting properties of the vector-producing cell, how MSCs naturally seek out tumors should be investigated in more detail. In addition, the localization, stability, and vector-producing capacity of gene-manipulated MSCs should be adequately analyzed *in vivo*. Tracking the localization of the MSCs may also help diagnose the recurrence of the disease. Such tracking may be performed by using a molecular imaging technique with MRI. To this end, it has been shown that MSCs labeled with fluorophore particles (IFPs) provide MRI contrast *in vivo* (24). Thus, this type of technology would enable us to

closely study MSC retention, engraftment, and migration in the clinic.

Although previous studies have illuminated the exciting possibilities of suicide cancer gene therapy, in most cases the therapies that were used delivered rather limited clinical benefits. For the sake of safety as well as improving the therapeutic effect of suicide cancer gene therapy, it is important that the suicide gene-expressing vector is accurately delivered to the tumor. This may be achieved by using MSCs to initiate virus production near tumor cells *in situ*. These viruses then transduce the tumor cells, which themselves produce virus progeny, thereby amplifying the transgene expression of the tumor. While the therapeutic benefit of this strategy remains to be tested in various orthotopic or metastatic tumor models, it may be promising for detecting and eradicating evasive tumors *in vivo*.

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Abbreviations: MSCs: mesenchymal stromal cells; GCV: ganciclovir; EPCs endothelial progenitor cells

Key Words: Cancer gene therapy, Multipotent mesenchymal stromal cells, Vector, Review

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Vector-producing tumor-tracking multipotent mesenchymal stromal cells for suicide cancer gene therapy

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Abstract

Suicide cancer gene therapy with retroviral vector-producing cells was in the way of an adjuvant to the surgical resection of recurrent glioblastoma, although any benefit appeared to be marginal. Further evaluation of the cancer gene therapy strategy with the vector-producing cells must incorporate improved delivery of vectors and transgenes to the tumor cells. We have previously demonstrated the ability of vector-producing tumor cells engineered by the adenovirus-retrovirus hybrid vector to destroy satellite tumor cells, although therapeutic efficacy for aggressive tumor has to be further evaluated by the systemic delivery of the vector-producing cells. Multipotent mesenchymal stromal cells (MSCs) appear to be effective delivery vehicle to seek out tumor cells *in vivo* and transport cancer-killing gene or immune products with minimal rejection reaction by the host. Therefore, MSCs-mediated tumor transduction with progeny vector production to improve suicide gene therapy might be feasible, if MSCs are capable of producing the recombinant viruses. Although therapeutic benefit in the various orthotopic or metastatic tumor models has to be further validated, this transduction strategy would realize systemic administration of the therapeutic vehicles to detect and eradicate evasive tumors *in vivo*.
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Keywords: cancer gene therapy, multipotent mesenchymal stromal cells, vector

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Suicide cancer gene therapy with vector producing cells

Antiglioma gene therapy with cells secreting a retroviral vector expressing the herpes simplex thymidine kinase (HSV-*tk*) transgene has been developed (1). This treatment can generate a significant local antitumor effect mediated by tumoricidal metabolites generated by HSV-*tk* activation of the prodrug ganciclovir (GCV). A factor that may enhance the therapeutic value of this technique is the bystander effect, where transduced tumor cells may communicate the apoptosis signal to neighboring cells. However, clinical benefit of this cancer gene therapy system was limited due to the poor efficiency of gene transfer (2). To improve the therapeutic potential of this system, it is necessary to enhance the efficiency of the therapeutic gene delivery *in vivo* as well as to increase the stability of the vector-producing cells.

We have previously described a hybrid vector system that uses the adenoviral vectors to deliver the retroviral vector and packaging proteins into cells (3). The system benefits from the efficient gene transfer characteristics of adenoviral vectors as well as the stable and long-term gene expression that is

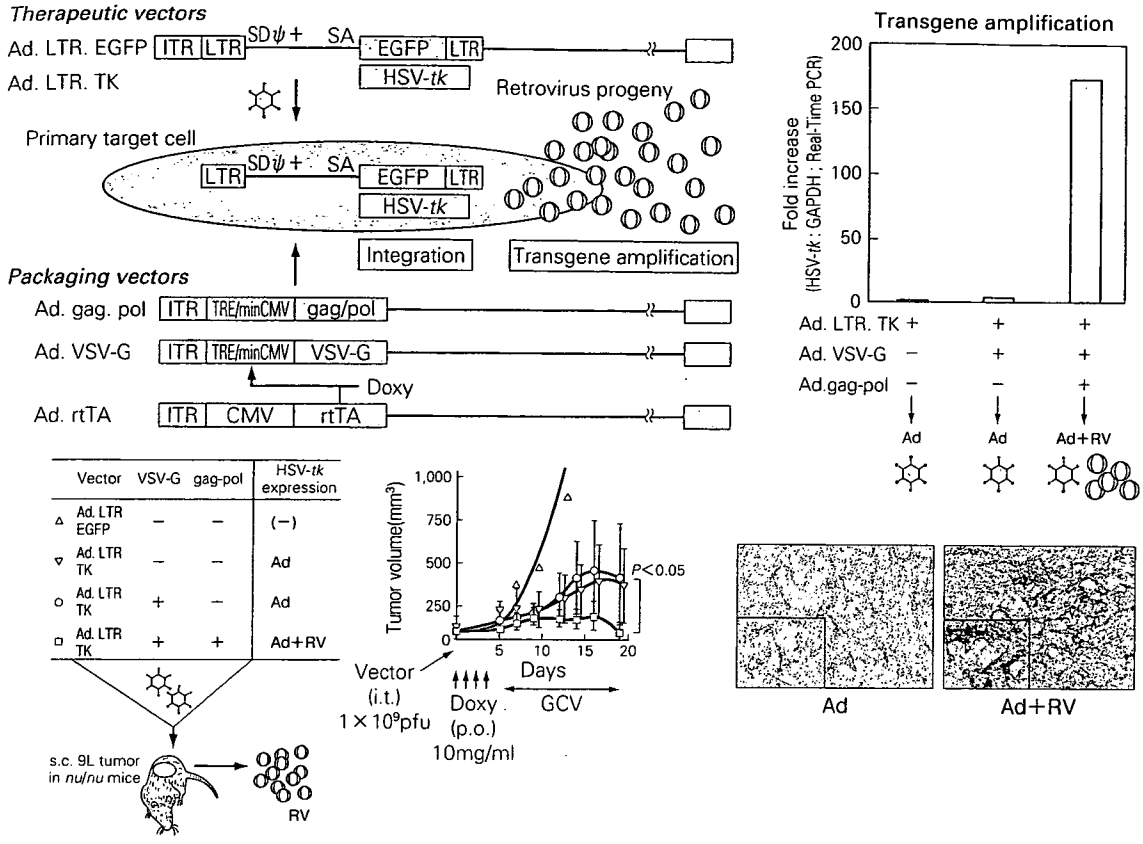

Figure 1 The adeno-retroviral hybrid vector containing the retroviral vector genome was constructed (Ad.LTR.EGFP or Ad.LTR.TK). Co-transduction of rat 9L glioma cells with adeno-retroviral hybrid vector together with vectors expressing packaging proteins of retroviruses (Ad.gag.pol, Ad.VSV-G) as well as an inducer (Ad.rtTA) increased the transduction efficiency. Injection of established subcutaneous 9L tumors on athymic mice with a combination of Ad.LTR.TK and packaging vectors followed by GCV treatment resulted in complete tumor regression in 50% of tumors at day 22, while no tumor regression was observed in control animals. Furthermore, the relative copy number of the HSV-*tk* gene in tumors treated with the adeno-retroviral vectors was significantly higher than that in control tumors. *In situ* hybridization suggested dispersion of the HSV-*tk* product across a wider area of tumor than in control tumors, which indicates the spread of the *in situ* generated retroviruses.

typical of the retroviral vectors. The initial co-transduction of primary target tumor cells with the adeno-retroviral hybrid vectors results in the transient production of recombinant retrovirus particles that then subsequently transduce neighboring tumor cells. Adenovirus vectors expressing transcomplementing genes for retroviral proteins and retroviral vector RNAs have been successfully used for the *in situ* transduction of tumors with enhanced therapeutic effects [Figure 1]. A single-step transduction of glioma cells with trans-complementing hybrid adeno-retroviral vectors effectively turned these cells into retrovirus vector producing cells, which in turn facilitates the transduction of adjacent cells. Taking this strategy to the next phase, application of cells with tumor-tracking properties as the vector producing cells would be promising for targeted spatial distribution of the therapeutic gene *in vivo*.

Multipotent mesenchymal stromal cells (MSCs) as a platform for vector production *in situ*

We propose here an improved *in situ* vector production strategy that efficiently produces the progeny vectors based on retrovirus or

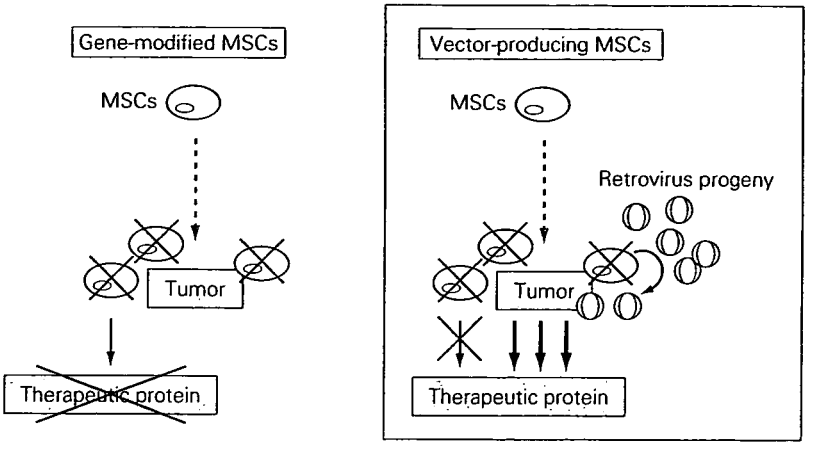

Figure 2
Left panel; Gene-modified MSCs
Although gene-modified MSCs have tumor-seeking property, local expression of the therapeutic protein is up to the presence of the MSCs.
Right panel; Vector-producing MSCs
Retrovirus progeny produced by the MSCs can transduce tumor cells *in situ* to amplify the therapeutic genes with extended expression of the therapeutic protein, even when MSCs fade away.

others [Figure 2]. To render the tumor-tracking properties for the vector-producing cells, important types of adult stem cells can be considered. The fibroblast-like plastic-adherent cells isolated from bone marrow and other sources have come to be widely known as mesenchymal stem cells or multipotent mesenchymal stromal cells (MSCs) (4). The tumor-seeking property of MSCs has been demonstrated, although mechanisms are poorly understood (5). This engraftment may be mediated by the cytokines released from the tumor or inflammatory tissue, such as hepatocyte growth factor (HGF) (6), vascular endothelial cell growth factors (VEGF) (7), transforming growth factor (TGF) (6), fibroblast growth factor (FGF) (6), platelet-derived growth factors (PDGF) (8), monocyte chemoattractant protein-1 (MCP-1) (9), and IL-8 (9). Chemokine C-X-C motif receptor 4 (CXCR4), although presents at the surface of a subset of MSCs, is important for mediating specific migration of MSCs to bone marrow (10). CXCR4 is known to govern cellular migration and homing in a variety of cell types, including neuronal and glial precursors in the developing brain. The only known chemokine that binds with CXCR4 is stromal-cell derived factor-1 (SDF-1). While CXCR4 itself is a major chemokine receptor on glioma cells and mediates their survival (11) , high-grade gliomas have recently been found to secrete significant levels of SDF-1 (12). SDF-1 alpha stimulates human glioblastoma cell growth through the activation of both extracellular signal-regulated kinases 1/2 and Akt. Therefore, CXCR4 would render MSCs with homing activity to the glioma with growth stimulation. The pattern of organ distribution suggested that infused cells were efficiently arrested in microvasculature during first-pass (13). Furthermore, these cells are not immunogenic and escape recognition by alloreactive T cells and natural killer cells (14). If MSCs efficiently integrate into human gliomas after intravascular or local delivery, MSCs can be exploited to take therapeutic advantage of vector production *in situ*. Engraftment

of MSCs into the tumor is also important to maintain their stem cell properties *in vivo*.

Other candidates of cells with tumor-tracking properties as the vector producing cells are including endothelial progenitor cells (EPCs). EPCs were isolated from peripheral blood CD34, Flk-1, or AC133 antigen-positive cells, which are considered to include a hematopoietic stem cell population, and were shown to be incorporated into foci of neovascularization (15). VEGF promotes adult vasculogenesis by enhancing EPC recruitment and vessel formation at the site of tumor neovascularization (16). Magnetic resonance imaging (MRI) of magnetically labeled endothelial progenitor cells demonstrated trafficking to sites of tumor angiogenesis (17).

Genetic manipulation of MSCs to produce progeny vectors

MSCs exhibit senescence-associated growth arrest and phenotypic changes during long-term *in vitro* culture. Overexpression of human telomerase reverse transcriptase (hTERT) in MSCs reconstitutes telomerase activity and extends life span of the cell (18). Telomerization of MSCs by hTERT overexpression maintains the stem cell phenotype of MSCs and it may be a useful tool for obtaining enough number of cells with a stable phenotype for cell differentiation studies and tissue engineering protocols.

Comprehensive assessment of the capability for the vector production with MSCs should be evaluated by the transduction of the MSCs with the viral components. Viral-based transduction techniques are efficient systems to deliver DNA into stem cells because they show high gene transduction and transgene expression in many cellular models. Various virus-based vectors, such as oncogenic retrovirus vectors or lentivirus-based vectors have been attempted to transduce MSCs. However, the use of integrating viral vectors has several disadvantages mainly involving safety risks. However, non-viral methods are rather inefficient for most primary cells. Nucleofection is an efficient non-viral transfection technique for MSCs, which then may be used as cellular vehicles for the delivery of biological agents (19). The Nucleofector technology, a non-viral electroporation-based gene transfer technique, seems to be promising as an efficient tool for transfecting MSC to produce progeny virus.

Future direction

Here we have proposed a feasibility of the vector-producing tumor-tracking cells to improve suicide cancer gene therapy. This strategy with MSCs would produce sufficient levels of vectors *in situ* for the killing of solid tumors. Furthermore, transient transduction of the MSCs with the adenovirus-based hybrid vector infection in a large-scale preparation of the VP-MSCs should realize the clinical investigation. The adenovirus-based hybrid vector with vectors expressing retroviral proteins can efficiently transduce cells to produce progeny vectors (3). In this context, the absence of the Coxsackie adenovirus receptor (CAR) on the MSCs seems to be a bottleneck (20). To overcome this issue, a chimeric Ad5 vector containing an Ad35 fiber (21) or a fiber-modified Ad5 vector containing an RGD-motif peptide in the HI loop of the fiber knob domain (22) was attempted to increase transduction efficiency. Alternatively, an adaptor molecule to bridge the gap between the viruses and MSCs may be applicable. We have previously developed a CAR-SCF fusion protein to improve transduction efficiency of the c-kit positive cells with the adenovirus vector (23). Similar CAR-ligand adaptor molecule would be of use to enhance transduction of the MSCs with the adenovirus vector.

For the improved tumor-targeting properties, mechanistic insight of the tumor-seeking

property should be further studied in detail. Besides, localization, stability, and vector-producing capacity of the gene-manipulated MSCs should be adequately analyzed *in vivo*. Localization of the cells should be also informative to diagnose the recurrence of the disease. A molecular imaging technique with MRI would be of great interest to track the distribution of MSCs. MSCs labeled with fluorophore particles (IFPs) provided MRI contrast *in vivo* (24). This type of technology would permit effective studies of MSCs retention, engraftment, and migration in the clinical investigation.

Although previous studies of cancer gene therapy have brought forward various exciting achievements, clinical benefits have been limited in most cases. Since safety is indispensable, effective vector delivery to the tumor with targeting machinery is important to improve therapeutic effects. Employment of MSCs to support virus production *in situ* should be functional to enhance therapeutic effects via the transgene amplification in the tumor. Although therapeutic benefit in the various orthotopic or metastatic tumor models has to be further validated, this transduction strategy would realize systemic administration of the labeling vehicles to detect and eradicate evasive tumors *in vivo*.

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Current Drug Targets and Future Therapy of Pulmonary Arterial Hypertension

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Abstract: During the last few decades, we have witnessed major improvements in the therapy of pulmonary arterial hypertension (PAH). PAH is characterized by abnormal remodeling of the pulmonary artery (PA) and increased PA pressures, resulting in a high premature mortality. Intravenous epoprostenol was the first effective approach toward improving the symptoms and survival of PAH patients. New prostanoids have also exhibited substantial clinical benefits; however, their long-term effects are under investigation. Endothelin-receptor antagonists and sildenafil have increased the lineup of therapeutic options against PAH. Combination therapy using these drugs is promising and is currently undergoing scrutiny in large clinical trials. An extensive analysis of the molecular mechanisms of PAH will produce novel targeted therapies. Most of the promising molecules target the inflammatory and proliferative processes underlying pathological PA remodeling. Interestingly, drugs used for other diseases, such as statins, Rho-kinase inhibitors, imatinib mesylate, may control the pathological vascular remodeling of PAH. Gene and cell therapy using vectors expressing prostacyclin synthase, endothelial nitric oxide synthase, or vascular endothelial growth factor are also promising strategies. However, the efficacy and safety of these approaches should be further tested in clinical trials. Genetic studies revealed some crucial genetic dispositions of familial PAH, although their pathobiological roles have not yet been fully clarified. Collaboration for integrated research will address these issues and generate greater clinical benefits for PAH patients.

Keywords: Pulmonary arterial hypertension, treatment, prostacyclin, endothelin, sildenafil, gene therapy, inflammation, proliferation.

INTRODUCTION

Pulmonary hypertension (PH) is defined as a mean pulmonary arterial pressure greater than 25 mmHg at rest or greater than 30 mmHg during exercise. Pulmonary arterial hypertension (PAH) is a disease characterized by abnormal remodeling of the pulmonary arteries (PAs), leading to increased pulmonary vascular resistance and right-sided heart failure. PAH can present in an idiopathic form (idiopathic PAH; IPAH); this occurs in women more often than men (>2:1), has a mean diagnostic age of 36 years, and is generally fatal within 3 years if untreated [1]. Modern treatment has markedly improved physical function and prolonged the survival of PAH patients. However, the factors responsible for disease initiation and progress remain unclear.

New studies on the molecular basis of PAH will open new windows to targeted therapies. This review provides a comprehensive overview of the rapidly expanding literature in this field, focusing on evidence-based treatment, novel drug targets, and future perspectives in gene and cell therapy. Here, we focus on issues concerning IPAH and PAH associated with connective tissue diseases, because these forms of PAH are the most well-described and studied.

1. CLASSIFICATION

The current classification of PAH, as established during the 2003 World Symposium on Pulmonary Arterial Hypertension, is depicted in Table **1** [2]. PAH consists of distinct disorders, including IPAH and familial PAH (FPAH), as well as PAH associated with various conditions such as connective tissue disease, congenital heart disease, portal hypertension, HIV infection, ingestion of drugs or toxins, and so on.

Table 1. Current Diagnostic Classification of Pulmonary Hypertension (PH)

1. Pulmonary arterial hypertension (PAH)
1.1. Idiopathic
1.2. Familial
1.3. Associated with
1.3.1. collagen vascular disease
1.3.2. congenital left-to-right shunt
1.3.3. portal hypertension
1.3.4. infection with HIV
1.3.5. drug and toxins
1.3.6. other conditions
1.4. Associated with substantial venous or capillary involvement
1.4.1. pulmonary veno-occlusive disease
1.4.2. pulmonary capillary hemangiomatosis
1.5. Persistent pulmonary hypertension of the newborn (PPHN)
2. Pulmonary hypertension with left heart disease
3. Pulmonary hypertension associated with lung disease or hypoxemia or both
4. Pulmonary hypertension due to chronic thrombotic or embolic disease or both
5. Miscellaneous

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Besides the diagnostic classification, patients should be stratified according to their functional capacity (Table 2); this is important to determine prognosis and guide therapeutic efforts. The functional classification of PAH patients has been adopted from the New York Heart Association (NYHA) classification of left heart disease [3]. At the time of diagnosis, approximately 80% of the patients present in class III or IV because PAH is notoriously overlooked. Since most clinical trials have focused on class III or IV patients, these data are not readily transferable to patients with less advanced disease. Earlier detection and adequate clinical classification of the disease will be undoubtedly important for clinicians.

Table 2. Functional Classification of Pulmonary Arterial Hypertension (PAH)

Class	Description
I	PAH without a resulting limitation of physical activity. Ordinary physical activity dose not cause undue dyspnea or fatigue, chest pain, or near syncope.
II	PAH resulting in a slight limitation of physical activity. The patient is comfortable at rest, but ordinary physical activity causes undue dyspnea or fatigue, chest pain, or near syncope.
III	PAH resulting in a marked limitation of physical activity. The patient is comfortable at rest, but less than ordinary physical activity causes undue dyspnea or fatigue, chest pain, or near syncope.
IV	PAH resulting in an inability to carry out any physical activity without symptoms. The patient has signs of right hear failure. Dyspnea, fatigue or both may be present even at rest, and discomfort is increased by any physical activity.

2. CURRENT DRUG THERAPIES OF PAH

The field of PAH has been advancing rapidly in recent years, and the pace continues to accelerate. Many important contributions have fueled our understanding of the pathogenesis of PAH (Fig. 1). Here, we summarize the current therapeutic strategies endorsed by recent clinical trials and describe problems encountered in daily practice.

Several consensus conferences have recently formulated evidence-based recommendations for the therapy of PAH. The current treatment algorithm is shown in Fig. 2. This algorithm is restricted to patients in class III or IV, because there are insufficient data to make recommendations for class I or II patients. Following the diagnosis of PAH, the first step of therapy, including general care, oral anticoagulation, diuretics, and oxygen should be initiated. However, these conventional therapies are associated with insufficient long-term prognosis when used alone [4]. Although the rationale of oral anticoagulation is based on the thrombophylic predisposition and thrombotic changes in the PA, the evidence for favorable effects of this treatment for IPAH patients is based on retrospective analysis of single center studies [5]. Furthermore, the risk/benefit ratio should be carefully considered in PAH patients with scleroderma or portopulmonary hypertension that may cause gastrointestinal bleeding. In contrast, PAH patients receiving chronic intravenous epoprostenol treatment are anticoagulated partly because of the additional risk of catheter-associated thrombosis.

Prior to the initiation of targeted treatment, the indication of calcium channel blockers (CCBs) should be assessed by vasoreactivity testing that is performed using a right heart catheter and short-acting vasodilators such as inhaled nitric oxide (NO) or intravenous epoprostenol [6, 7]. Based on a large database, a positive acute response to vasodilators is defined as a decline in mean pulmonary arterial pressure by at least 10 mmHg to <40 mmHg in the presence of normal cardiac output [6, 7]. Less than 10% of IPAH patients fulfill the criteria and derive sustained clinical benefits from treatment with CCBs such as nifedipine, diltiazem, and amlodipine [8]. The evidence for the favorable effects is based on the results from single center non-randomized, non-controlled trials. Since no study has determined which of the CCBs is maximally efficacious in controlling of PAH, the patient's heart rate at baseline may provide a possible selection criteria: relative bradycardia favoring nifedipine, and relative tachycardia favoring diltiazem. The CCB treatment should be initiated with reduced doses to be increased cautiously and progressively to the maximal tolerated regimen. Limiting factors for dose increase are generally systemic hypotension and lower limb peripheral edema.

Inhaled NO has selective and potent pulmonary vasodilator effects during short-term treatment of adult and newborn PAH. For chronic PAH patients, however, inhaled NO has been primarily used for acute vasoreactivity testing, because its long-term efficacy and safety remain unclear [8]. Augmentation of endogenous NO production with substrate (L-arginine) supplementation may also be effective for PAH patients. Despite growing use of oral L-arginine, there are no rigorous randomized clinical trials to assess its long-term effects [8]. Increased serum concentration of polyamines, which are proproliferative, may raise concern about potential adverse effects of L-arginine.

IPAH patients those who do not meet the acute vasoreactivity criteria should receive the endothelin (ET)-receptor blocker bosentan, inhaled iloprost, or subcutaneous treprostinil. Patients who present with or progress to class IV are candidates for intravenous epoprostenol treatment. Although current data supporting this concept are limited, combination treatment is being endorsed as a therapeutic option in patients not responding to monotherapy.

2.1. Prostanoids

Endogenous prostacyclin (PGI_2) is a potent vasodilator with antiplatelet activity. The synthesis of PGI_2 is markedly diminished in the pulmonary endothelium of PAH patients. Many studies support the evidence that a continuous supplement of sufficient amounts of prostanoids plays an essential role in current PAH therapy (Fig. 3).

2.1.1. Intravenous Epoprostenol

The introduction of intravenous epoprostenol treatment, originally used as a bridge to lung transplantation, is a major advancement in the management of patients with severe PAH. A randomized controlled trial exhibited long-term improvements in hemodynamics, exercise capacity, and survival in epoprostenol-treated patients [9]. The effects of epoprostenol may go far beyond pulmonary vasodilatation and inhibit PA remodeling by regulating the proliferation of pulmonary arterial smooth muscle cells (PASMCs) as well

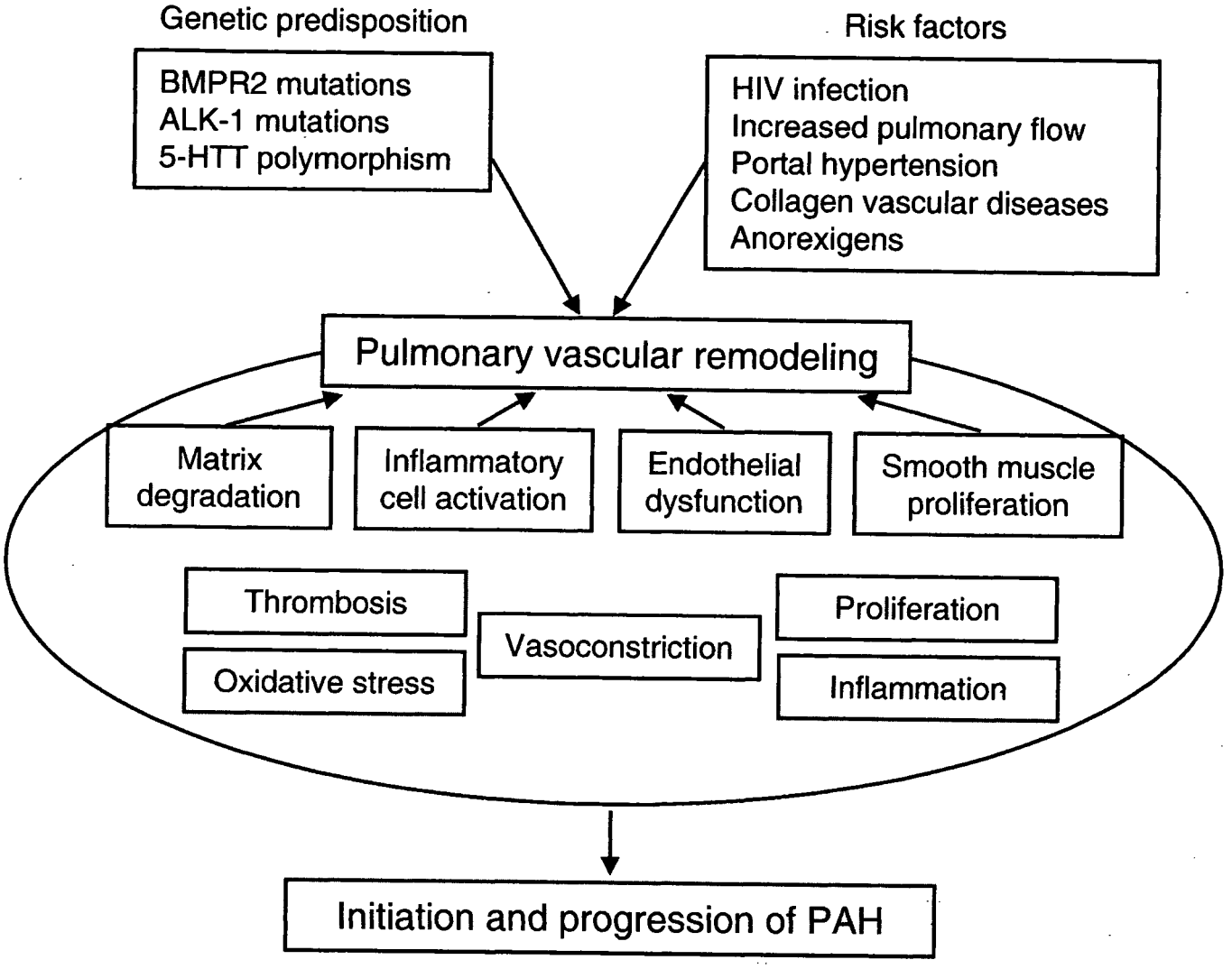

Fig. (1). Potential pathogenesis of pulmonary arterial hypertension (PAH). BMPR2: bone morphogenetic protein receptor 2, ALK-1: activin-receptor-like kinase-1, 5-HTT: serotonin transporter gene.

as the expression of proinflammatory cytokines and chemokines [10-12]. Despite an improvement in survival, the long-term prognosis remains unsatisfactory with a 3-year survival rate of about 63% in functional class III/IV patients treated with epoprostenol [13, 14]. This situation has encouraged researchers to find a new strategy to prolong the survival of patients with severe PAH.

The epoprostenol treatment has major problems in its delivery system. Because of its short biological half-life (2–3 minutes), epoprostenol must be administered with continuous intravenous infusion system consisting of a portable pump and a central venous catheter. Pump failure, catheter dislocation, and sepsis may cause life-threatening complications. In addition, severe adverse effects can occur with overdosage of the drug. Acutely, overdosage can lead to systemic hypotension. Long-term overdosage can lead to the development of hyperdynamic state and high-output cardiac failure. Abrupt or inadvertent interruption of the infusion results in a rebound worsening of the symptoms and even death. Although common side effects of this therapy include headache, flushing, nausea, erythema, and muscle pain, these tend to be dose dependent and often respond to a cautious reduction in dose. New approaches such as inhaled iloprost or subcutaneous injection of treprostinil are expected to be useful alternatives because they have longer biological half-lives, resulting in easier handling and fewer complications. Since the long-term efficacy of these new approaches is still unsatisfactory, it is desirable to develop approaches that are more effective.

2.1.2. Inhaled Iloprost

Iloprost is a PGI_2 derivative with a pharmacodynamic profile that is very similar to epoprostenol; however, it has a longer serum half-life of 20–30 minutes. Since the acute hemodynamic effects of a single iloprost inhalation disappear after 45–60 minutes, patients have to inhale 6–12 times per day to cover 24 hours. The Aerozolized Iloprost Randomized (AIR) study, a 12-week placebo-controlled trial involving 207 PAH patients, found a significant improvement in functional class, exercise capacity, and hemodynamics in those who received iloprost [15]. Overall, inhaled iloprost was well tolerated; cough, flushing, and headache oc-

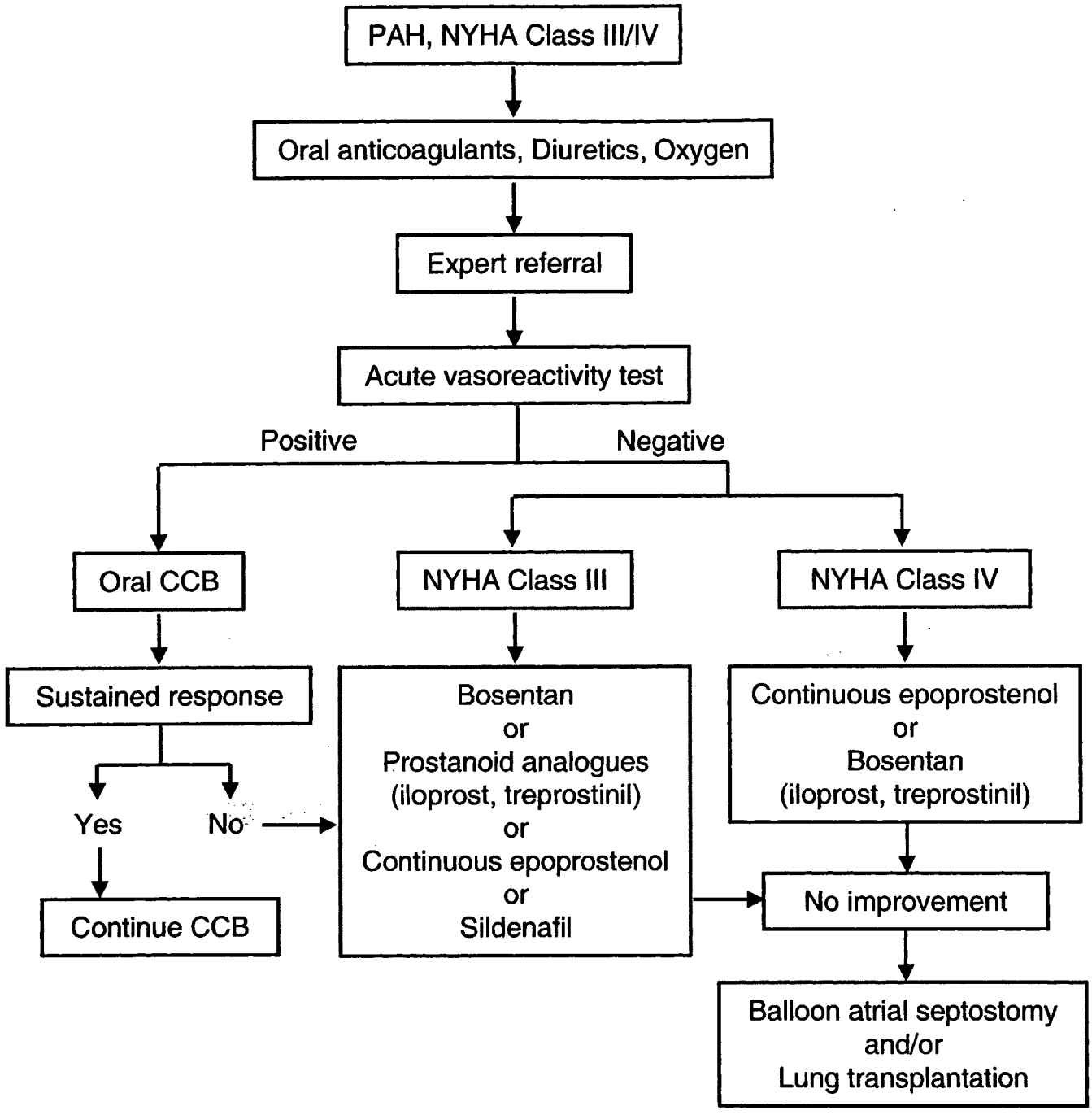

Fig. (2). Treatment algorithm for human PAH. The algorithm is restricted to patients in NYHA class III or IV because they represent the largest population included in controlled clinical trials. PAH: Pulmonary arterial hypertension, NYHA: New York Heart Association, CCB: calcium channel blockers.

curred more frequently in the iloprost group. However, the long-term efficacy and safety of this regimen still needs to be evaluated by rigorous clinical trials.

2.1.3. Subcutaneous Treprostinil

Treprostinil is another stable PGI_2 derivative with a serum half-life of 30–45 minutes that leads to greater acute hemodynamic effects compared to epoprostenol in patients with PAH [16]. To circumvent the problems of continuous intravenous epoprostenol, subcutaneous infusion of treprostinil has been proposed as an alternative to the administration of prostanoids to patients with PAH. In the largest randomized placebo-controlled trial that has thus far been performed in 470 patients with PAH (functional class II-IV), a significant improvement in hemodynamics and the 6-minute walking distance was observed in patients receiving a 12-week treatment of treprostinil [17]. However, there were no differences in survival and other clinical outcomes in this study. Furthermore, approximately 85% of the patients undergoing this therapy experienced untreatable severe pain at

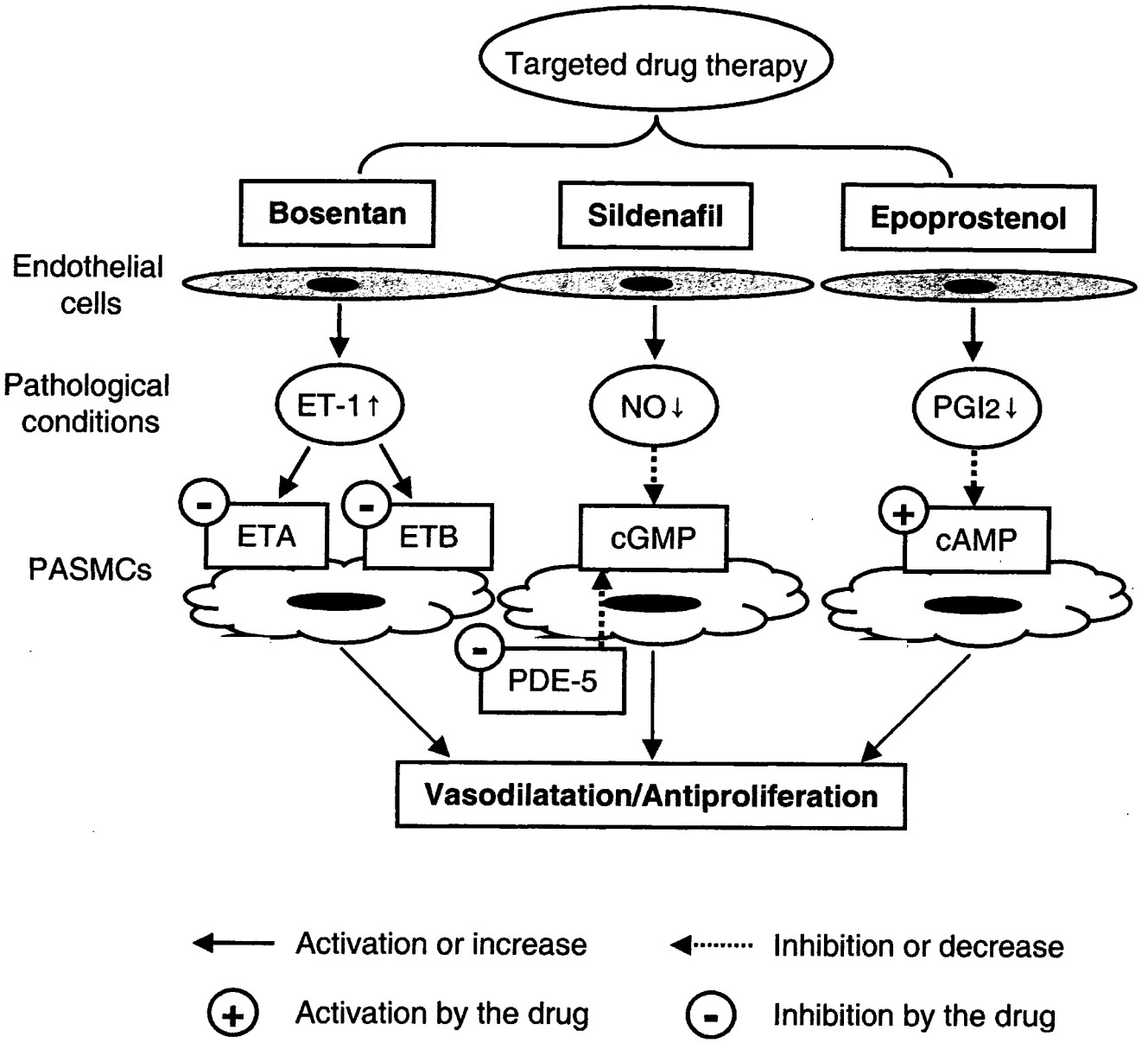

Fig. (3). Current drug targets of pulmonary arterial hypertension. PASMC: pulmonary arterial smooth muscle cell, ET-1: endothelin-1, ETA/B: ET-1 type A/B receptors, NO: nitric oxide, PGI2: prostacyclin, cGMP: cyclic guanosine monophosphate, cAMP: cyclic adenosine monophosphate, PDE-5: phosphodiesterase-5.

the infusion site. However, similar to inhaled iloprost therapy, treprostinil treatment poses the problem of unsatisfactory long-term efficacy. Therefore, treprostinil may be considered only when other options are not available.

2.1.4. Beraprost

Beraprost is an orally active PGI_2 analogue with a 30–45 minute serum half-life. This drug was introduced by Japanese groups who reported data from several uncontrolled trials suggesting some clinical benefits. However, the Arterial Pulmonary Hypertension And European Beraprost Trial (ALPHABET), the first randomized placebo-controlled trial that enrolled 130 patients (functional class II and III), exhibited a slight but significant increase in the 6-minute walking distance in the beraprost- treatment group and no difference in the hemodynamic findings [18]. The second randomized placebo-controlled trial that included 116 patients with PAH (functional class II and III) demonstrated short-term (3–6 months) but no long-term (9–12 months) beneficial effects of beraprost [19]. Both studies also reported drug-related adverse effects including headache, jaw pain, flushing, diarrhea, and palpitation. Based on these data, the drug has not received approval as therapy for PAH in the US and Europe.

2.2. Endothelin-Receptor Antagonists

Endothelin-1 (ET-1) is a potent vasoconstrictor and a mitogen for vascular smooth muscle cells (VSMCs) and fibroblasts. The ET-1 levels in plasma and pulmonary tissues are increased in patients with PAH and correlate with the severity of the disease and shortened survival [20, 21]. ET-1 is synthesized by endothelial cells (ECs) acting on two distinct G-protein coupled receptors (ET_A and ET_B) in an

autocrine/paracrine manner. ET_A receptors, primarily located on VSMCs, mediate vasoconstriction and cellular hypertrophy. ET_B receptors, located on ECs and VSMCs, mediate vasodilatation by liberating PGI_2 and NO. Several animal and clinical studies demonstrated a striking upregulation of ET_B receptors in pulmonary VSMC, indicating a specific role of the ET_B receptor in vasoconstriction and proliferation in vascular remodeling of PAH (Fig. **3**) [22, 23]. The superiority of selective ET_A antagonists or $ET_{A/B}$ dual antagonists in PAH therapy is still being debated because no clinical study comparing the two inhibitors has been performed.

2.2.1. Bosentan

Bosentan is an orally active dual $ET_{A/B}$ receptor antagonist, which was first approved for the treatment of PAH in the functional class III and IV. The first small randomized placebo-controlled trial that included 32 patients demonstrated significant improvements in the 6-minute walking distance, dyspnea index, functional class, and hemodynamics [24]. Based on these findings, the larger Bosentan Randomized Trial of Endothelin Antagonist Therapy (BREATHE-1) was launched; this included 213 patients with IPAH and PAH associated with connective tissue diseases [25]. The administration of bosentan demonstrated a significant increase of the 6-minute walking distance and the time to clinical worsening, with an improvement of the right heart function assessed by echocardiography [26]. Bosentan is well tolerated; nevertheless, it is recommended that the dose at initiation of therapy be 62.5 mg twice daily since administration at higher doses (125 mg or 250 mg twice daily) may cause a reversible increase in serum aminotransferases (4% or 14% of patients, respectively). Bosentan treatment may also be associated with the development of mild anemia, lower extremity edema, testicular atrophy, and tetratogenic effects. Monotherapy with bosentan produced insufficient effects on long-term survival. McLaughlin *et al.* [27] followed 169 PAH patients receiving bosentan as a first-line treatment for a mean period of 2.1 years. They reported that the survival rates were 96% at 1 year and 89% at 2 year, but 23% of the patients required the addition of or a transition to intravenous epoprostenol treatment and 1.7% of these underwent lung transplantation.

2.2.2. Sitaxsentan Sodium and Abbrisentan

Sixtasentan sodium is a selective ET_A receptor antagonist with a 6500-fold higher affinity for ET_A receptors than for ET_B receptors [28]. The drug is orally active with a long plasma half-life of 5–7 hours. The Sitaxsentan to Relief Impaired Exercise (STRIDE) trial including 178 patients with PAH showed significant improvements in the 6-minute walking distance and hemodynamics [28]. Mild elevation of serum liver aminotransferases was observed in the high-dose group (300 mg per day) but not in the low-dose group (100 mg per day). The most frequent adverse events with sitaxsentan treatment were headache, peripheral edema, nausea nasal congestion, and dizziness, reactions previously noted with ET-1 receptor antagonists. In addition, the sitaxsentan treatment exhibits prolongation of prothrombin time caused by the inhibition of CYP2C9 P450 enzyme. The STRIDE-II trial is currently testing safety and efficacy of the drug at doses of 50 or 100 mg per day. Ambrisentan—another selective ET_A-receptor antagonist with a 77-fold higher affinity for ET_A receptors than for ET_B receptors—is being studied with regard to PAH. These results regarding new ET_A-receptor antagonists will be available in the future.

2.3. Phosphodiesterase Type 5 Inhibitors

Phosphodiesterases inactivate cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). The vasodilatory signalings of PGI_2, NO, and atrial natriuretic peptide (ANP) are mediated by cAMP and cGMP. The isoform phosphodiesterase (PDE)-5 is abundantly expressed in the lung where it inactivates cGMP; however, in systemic circulation, it is inappreciably expressed. Thus, PDE5 inhibitors can specifically activate pulmonary cGMP and dilate the PA without affecting the systemic blood pressure (Fig. **3**). Moreover, these drugs can activate cAMP by reducing PDE3 activity, resulting in additional vasodilatation. These features make PDE5 inhibitors promising drugs for PAH treatment.

2.3.1. Sildenafil

Sildenafil is an orally active, selective PDE5 inhibitor with a 4-hour plasma half-life. It promotes NO-mediated vasodilatation and inhibits the proliferation of PASMC [29]. The Sildenafil Use in Pulmonary Arterial Hypertension (SUPER), a double-blind randomized placebo-controlled trial in which 278 patients with PAH (either idiopathic or associated with connective-tissue disease or with repaired congenital systemic-to-pulmonary shunts) were enrolled, revealed the clinical efficacy of the drug. The patients orally received sildenafil (20, 40, or 80 mg) three times daily for 12 weeks; this resulted in significant improvements in exercise capacity, functional class, and hemodynamics [30]. Although the incidence of clinical worsening did not differ significantly between the sildenafil group and the placebo group, the drug was shown to be highly tolerable without causing serious adverse events; minor side effects include headache, nausea, nasal congestion, and visual disturbances. Among the 222 patients completing 1-year treatment with sildenafil monotherapy, the improvement from baseline at 1 year in the 6-minute walking distance was 51 m, while previously reported improvements achieved by the administration of intravenous epoprostenol [9], inhaled iloprost [15], oral bosentan [25], and subcutaneous treprostinil [17] were 47 m, 36 m, 44 m, and 16 m, respectively. Sildenafil was approved for PAH therapy by the US FDA in 2005 and will be a promising first-line agent for functional class II and III patients.

2.3.2. Other PDE5 Inhibitors

Vardenafil and tadalafil act as preferential, but not fully selective inhibitors of PDE5. Although both drugs are approved for erectile dysfunction, they have not been sufficiently evaluated for use in PAH therapy. Ghofrani *et al.* [31] reported that sildenafil, vardenafil, and tadalafil markedly differ in their pulmonary vasorelaxant activity, tissue selectivity, and pharmacokinetics. Interestingly, Aizawa *et al.* [32] have recently reported a pilot study that demonstrates the efficacy and safety of long-term oral vardenafil therapy for patients with PAH and chronic pulmonary thromboembolism. Further clinical studies are needed whether the preferable results of sildenafil will be transferable to other PDE5 inhibitors.