cadherin, a specific endothelial cell–cell adhesion molecule, we observed VE-cadherin at cell–cell junctions (Figs. 3C, D).

Late EPCs, but not early EPCs, participated in tube formation with HUVECs

When EPCs are mixed with HUVECs and incubated on Matrigel, EPCs are incorporated into the tubular structure formed by HUVECs [5]. To confirm this, early EPCs or late EPCs were mixed with HUVECs and they were applied to the same tube formation systems. To distinguish the EPCs from HUVECs, the EPCs were labeled with PKH26 Red. Tubular structures formed by these EPCs and HUVECs were then labeled with Calcein-AM. The fluorescence of EPC and HUVECs was observed with a laser confocal microscopy. The early EPC as well as the late EPCs were observed in the tubular structure. However, by a three-dimensional imaging, we demonstrated that the early EPCs were only attached to the tube surface and were not incorporated into the tube structure formed by HUVECs (Fig. 4A). In contrast, the late EPCs participated in tube formation with HUVECs (Fig. 4B). To quantify the incorporation of EPCs into tube structure was calculated by the area of EPC per the indicated length of tubular structure. The incorporation of late EPCs into tubular structures was over 5-fold larger than that of early EPCs (Fig. 4C).

Late EPCs integrated into pre-existing tubular structures formed by HUVECs while early EPCs caused tubular sprouting

In order to investigate whether EPCs could substitute for HUVECs in preformed tubular structures, EPCs and HUVECs were independently cultured. HUVECs labeled with PKH26 Red were seeded onto the substrate and EPCs labeled with PKH26 Green were suspended in Matrigel. The patterned HUVECs on the substrate were transferred to the EPC-containing Matrigel. The late EPCs migrated toward the tubular structures and were observed adjacent after 6 h (Fig. 5A) and attached to the tubes 10 h later (Fig. 5B). Thereafter, they were incorporated into the tubular structure 24 h later (Fig. 5C). In contrast, early EPCs did not migrate toward the tubes and were not

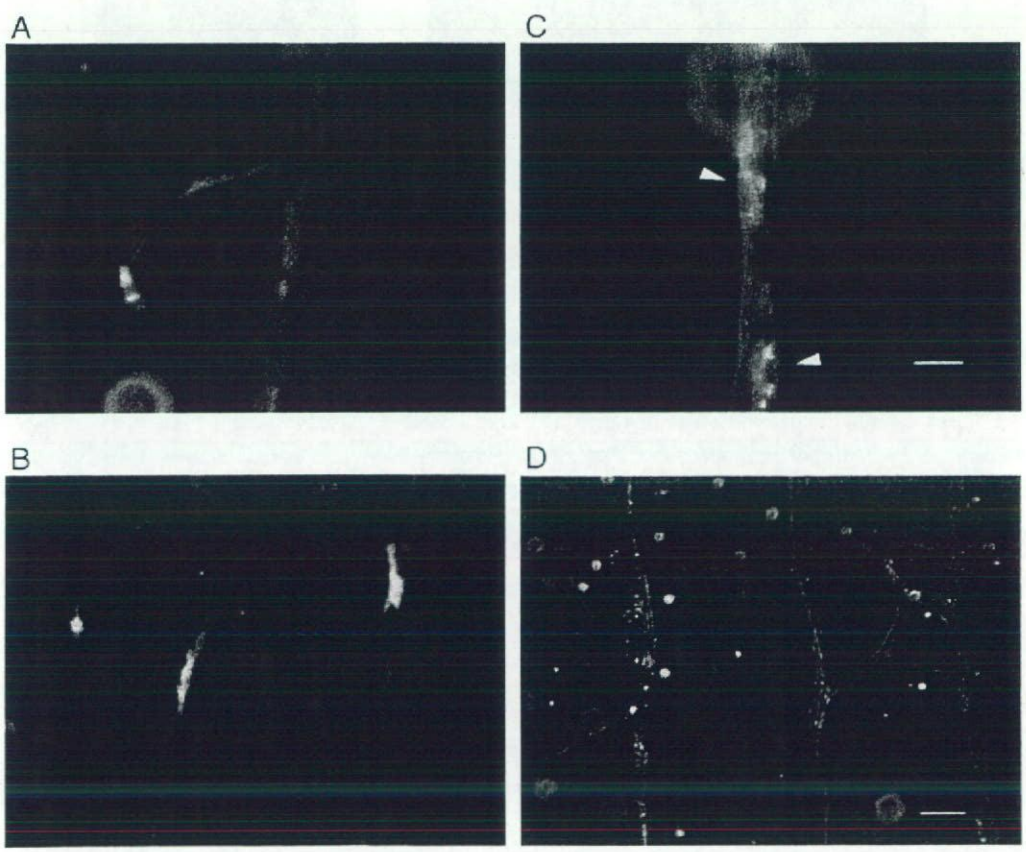

Fig. 5 – Incorporation of EPCs into preformed tubular structures. HUVECs labeled with PKH26 Red were seeded on the substrate and EPCs labeled with PKH26 Green were suspended in Matrigel. The patterned HUVECs on the substrate were transferred to the EPC-containing Matrigel. (A) Six hours after transplantation, late EPCs were migrating toward the tubular structure preformed by HUVECs. (B) Ten hours after transplantation, late EPCs were attached to the tube. (C) Twenty-four hours after transplantation, late EPCs were incorporated into the tubular structure preformed by HUVECs (yellow arrow heads). Scale bar = 30 μm. (D) Twenty-four hours after transplantation, early EPCs had not been incorporated into the tubular structure preformed by HUVECs. The HUVECs in the tubular structure were sprouting and migrating. Scale bar = 100 μm.

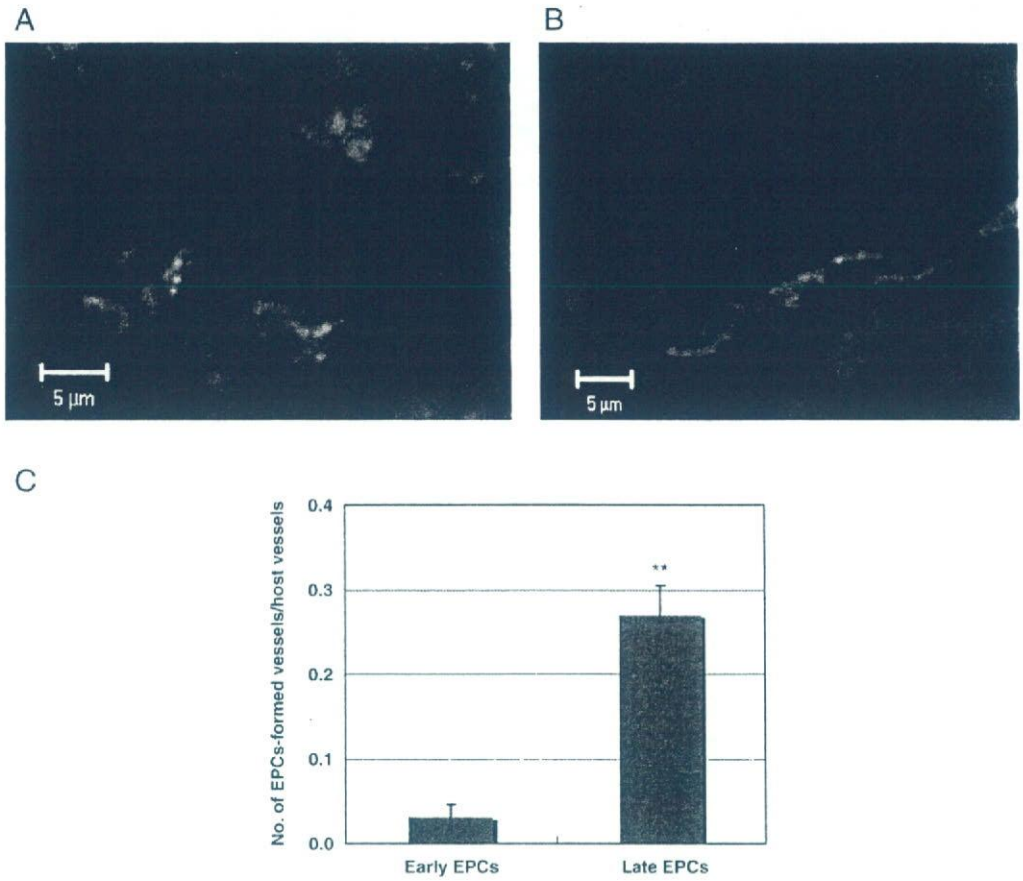

Fig. 6 – *In vivo* angiogenesis assay. After occlusion of murine auricular vessel, early (A) or late (B) EPCs labeled with PKH26 Red were subcutaneously injected into the occluded pinna. Forty-eight hours after the cell injection, the mice were given BS1-lectin (green) intravenously and sacrificed. Scale bar = 5 μm. (C) The numbers of blood vessels derived from each EPCs observed in 5 sections were normalized to the number of recipient blood vessels. The data are shown as the mean and SE of three mice and data are mean of 5 fields/mouse. $^{**}P < 0.01$ vs. early EPCs.

incorporated during the observation period. However, after HUVECs were transferred to Matrigel containing early EPCs, the HUVECs in the tubular structures initiated sprouting and migrated toward early EPCs as evidenced by branching patterns between the tubular structures (Fig. 5D).

In vivo angiogenesis assay

To confirm the tube forming activity of each EPCs *in vivo*, the vessels derived from implanted EPCs were observed in the ear vessel occlusion model, early or late EPCs labeled with PKH26 Red were injected into the occluded pinna. Blood vessels derived from the late EPCs were observed in the pinna, but only a few blood vessels derived from early EPCs were observed (Fig. 6).

Discussion

We investigated the tube-forming capacity of early EPCs and late EPCs isolated from human peripheral blood and umbilical cord blood, respectively. In the previous report, it was demonstrated that late EPCs were enriched in umbilical cord blood compared with adult peripheral blood [23]. Therefore, we isolated late EPCs from human umbilical cord blood. We used our novel method for capillary engineering which makes use of photo-catalytic lithography. Traditional assays for assessing characteristics of tube formation *in vitro* have been carried out by cultivation of endothelial cells in type I collagen and Matrigel. However, these methods could not distinguish between tube formation and morphological changes in the cells. The luminal structure of the vascular tube made in our present method was confirmed by an electron microscopy, a confocal laser microscopy and dye microinjection [22]. This technique is unique because the method allows one to focus on the process of tube formation. In contrast, tube formation in collagen and Matrigel evaluates the total activity of endothelial cells including migration, invasion and tube formation [24]. Using this novel method, we demonstrated that late EPCs participated in the formation of tubular structures with mature endothelial cells, i.e., HUVECs. It is interesting that late EPCs migrated toward and adjacent to pre-existing tubular structures and finally were incorporated

into the structure itself. In contrast, early EPCs could not form tubular structures, and they induced migration and sprouting of HUVECs present in the tubular structure. In a previous study using Matrigel, Hur et al. [18] demonstrated that early EPCs were incorporated into tubules when co-cultured with HUVECs, although the formation was weaker than that of late EPCs. However, that paper demonstrated the incorporation of EPCs into network structures of HUVECs on Matrigel, but could not show the incorporation of EPCs in tubular structures.

Our results showed that early EPCs were not capable of constructing tubular luminal structures even when they were co-cultured with mature endothelial cells. Instead, they stimulated the migration and sprouting of HUVECs from the tubular structure. These phenomena may be interpreted by the release of some growth factors from early EPCs. In our experiment, the amount of secreted interleukin-8 from early EPCs was significantly higher than that from late EPCs (0.81 ± 0.07 vs. 0.44 ± 0.02, ng/ml, $P<0.01$). It has been also reported that early EPCs secrete angiogenic factors such as VEGF [18,21], hepatocyte growth factor (HGF), and granulocyte-colony stimulating factor (G-CSF) [21]. These factors are known to stimulate endothelial cell migration and proliferation. In vivo experiments, implanted late EPCs in the occlusion tissues cause to construct new blood vessels by themselves, but early EPC could not. This result may be discussed with a hierarchy of EPCs [23]. It has been also reported that the different types of cells derived from peripheral blood have distinct actions in healing activity [25]. In this study, we demonstrated specific and distinct behaviors of early and late EPCs in tube formation and how they affected mature ECs in tube formation.

Although numerous reports have demonstrate that early EPCs have the potential to construct new blood vessels by themselves [5,6], some studies argued that early EPCs were not actually incorporated into newly formed vessels [26,27]. This discrepancy may be due to marked heterogeneity of EPCs used in their experiments. Numerous studies have fractioned EPC by phenotypic markers such as CD34, CD133, and VEGFR2 and various methods of EPCs isolation have been reported [5,6,28,29]. Among them CD34 has been often used for the marker of EPCs. However, Romagnani et al. showed that CD14 positive MNC-derived EPCs, which had been fractioned as CD34 negative, express very low level of CD34 using an antibody-conjugated magnetofluorescent liposomes (ACMFL) technique [30]. From these results, they suggested that $CD14^{+}$ $CD34^{low}$ cells are the major source of early EPCs obtained from human peripheral blood MNC. This subset exhibited clonogenicity and multipotency to differentiate not only into endothelial cells, but also into osteocytes, or neural cells [30]. This report suggests that it is difficult to sort EPCs into highly defined fractions by ordinary FACS technique. In fact, each EPC population in previous studies is supposed to have a different phenotype even from the same source. To avoid these complexities, we collected and isolated early and late EPCs by focusing on their characteristics such as morphologies and proliferation pattern, and compared their tube-forming activities in short duration *in vitro* assays.

While the regenerative potential of EPCs has been demonstrated in animal models of myocardial and limb ischemia, the number of EPC available for transplantation is very important for cell-based therapy. Because EPCs are derived from a limited endogenous pool, it is necessary to expand the number of EPCs *in vitro* or modulate phenotypes of EPCs. Iwaguro et al. reported that VEGF gene transfer in EPCs stimulated neovascularization in an *in vivo* model [31]. Murasawa et al. demonstrated that gene transfer of human telomerase reverse transcriptase into EPCs enhanced their angiogenic properties, mitogenic and migratory activities, and cell survival [32]. From the standpoint of cellular proliferation and phenotypic stability, late EPCs are superior to early EPCs. Therefore, the use of late EPCs for tissue engineering has been challenged [33–35]. With our method of generating transplantable capillary networks, the formation of tubular structures *in vitro* is a necessary precondition. The data presented here are thus important since the results demonstrate that late EPCs are a candidate for tissue engineering.

In conclusion, our data provide the first definitive evidence that early EPCs promote angiogenesis through migration and proliferation of mature endothelial cells, whereas late EPCs can form blood vessels. These results suggest that early EPCs and late EPCs have different roles in neovascularization *in vivo*. Finally, we expect that the novel culture system using a patterned substrate might be useful for future *in vitro* analyses of neovascularization.

Acknowledgments

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ARTICLES

薬の名前
ステムを知れば薬がわかる

Stems used in drug names : For the better understanding of pharmacological actions of drugs

第18回

国立医薬品食品衛生研究所
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はじめに

本連載第17回(本誌2007年12月号)では，抗悪性腫瘍薬のステムとして，

「-sulfan」：メタンスルホネート系抗悪性腫瘍薬
「-mustine」：β-クロロエチルアミノ構造を持つ抗悪性腫瘍薬
「-tepa」：チオテパ系誘導体
「-ribine」：ピラゾフリン系リボフラニル誘導体
「-trexate」：葉酸類縁化合物
「-trexed」：チミジル酸合成酵素阻害薬
「mito-」：細胞核に対して毒性を有する抗悪性腫瘍薬

を紹介した。

今回は，生物薬品の第6回目として，酵素性医薬品のステムを紹介する。

「-ase」：酵素

「-ase」は，酵素(Enzyme)を示すステムである。「-ase」は，さらに「-uplase」(ウロキナーゼ型プラスミノーゲンアクチベーター)，「-teplase」(組織プラスミノーゲンアクチベーター)，「-diplase」(プラスミノーゲンアクチベーターと他の酵素との融合タンパク質)，「-lipase」(リパーゼ活性を持つ酵素)，「-dismase」(スーパーオキシドジスムターゼ活性を持つ酵素)などのサブステムに分けられる。

(1)「-ase」：タンパク質分解酵素

「-ase」は，タンパク質分解酵素(proteinase)の(サブ)ステムとしても使用される。タンパク質分解酵素を示すサブステム「-ase」を持ち，わが国で承認されている医薬品にKallidinogenase(カリジノゲナーゼ)，Serrapeptase(セラペプターゼ)，L-Asparaginase(L-アスパラギナーゼ)，Pronase(プロナーゼ)，Urokinase(ウロキナーゼ)およびTisokinase(チソキナーゼ)がある。このうち，カリジノゲナーゼ，セラペプターゼおよびウロキナーゼは日局収載品目である。

カリジノゲナーゼは，血液中のキニノーゲンに特異的に作用してキニンを遊離するキニノゲナーゼ(カリクレイン)の1種で，ブタ膵臓由来のものが医薬品として使用されている。遊離したキニンは，末梢血管の拡張なら

びに微小循環速度の亢進を介して血流増加作用を示す。わが国でカリジノゲナーゼは，高血圧症，メニエール症候群，閉塞性血栓血管炎(ビュルガー病)における末梢循環障害の改善および更年期障害，網脈絡膜の循環障害改善薬として適用されている。

セラペプターゼは，セラチア(*Serratia*)属細菌から精製したタンパク質分解酵素である。わが国では，手術後および外傷後，慢性副鼻腔炎，乳汁うっ滞における腫脹の緩解，ならびに気管支炎，肺結核，気管支喘息の喀痰喀出困難および麻酔後の喀痰喀出困難に適用される。

L-アスパラギナーゼは，321個のアミノ酸残基からなるサブユニット4つで構成されるタンパク質である。血中のL-アスパラギンを分解し，アスパラギン要求性腫瘍細胞を栄養欠乏状態にすることによって抗腫瘍作用を発揮する。日本および米国では急性白血病(慢性白血病の急性転化を含む)および悪性リンパ腫の治療に用いられている。

プロナーゼは，放線菌*Streptomyces griseus*が産生するタンパク質分解酵素である。わが国では，胃内視鏡検査における胃内粘液の溶解除去や消化異常症状の改善を目的に使用されている。また，イソプロテレノールとの混合剤が持続性気管支拡張・粘液溶解剤に適用されている。

その他「**-ase**」を持つ品目で，海外で承認されている医薬品にRasburi**case**(ラスブリ**カーゼ**)，Streptokin**ase**(ストレプトキ**ナーゼ**)，Pegaspar**gase**がある。

ラスブリカーゼは，*Saccharomyces cerevisiae*から産生される遺伝子組換え尿酸オキシダーゼである。悪性腫瘍あるいは化学療法に起因して発現する高尿酸血症治療薬として欧米で使用されている。わが国では2007年にJANに収載された。

Pegaspargaseは，大腸菌で産生されたL-アスパラギナーゼに，分子量約5,000のポリエチレングリコールを共有結合させたPEG化タンパク質で，米国で承認されている。なお，「**Peg-**」はポリエチレングリコール(PEG)が結合していることを意味する接頭語である(本連載第5回(本誌2006年12月号)参照)。

その他タンパク質分解酵素を示すサブステム「**-ase**」を持つ医薬品としてINNには，Brin**ase**，Ocr**ase**，Promel**ase**，Sferic**ase**が収載されている。

INNには「**-ase**」を持たないタンパク質分解酵素もいくつか収載されている。わが国で承認されている医薬品ではBromelain(ブロメライン)がある。ブロメラインは，パイナップルの果汁，または葉茎の搾汁より調製したタンパク質分解酵素で，手術後および外傷の腫脹の緩解，慢性気管支炎や気管支喘息の喀痰困難の改善，熱傷や化膿創などの創傷面の壊死組織の分解除去・洗浄およびそれに伴う治癒促進に用いられている。

「**-ase**」を持たないタンパク質分解酵素性医薬品としては，欧州で承認され，JAN収載品目でもあるChymotrypsin(キモトリプシン)や，米国で承認されているChymopapainもある。その他，INNにはBatroxobin，Defibrotide，Fibrinolysin(human)，Sutilainsが収載されている。

タンパク質分解酵素には血栓溶解系に関与するものもある。循環血液中には血栓溶解作用を持つプラスミンの前駆体であるプラスミノーゲンが存在する。循環血液中のプラスミノーゲンは，プラスミノーゲンアクチベーターによって560番目のアルギニンと561番目のバリンの間が切断されて，活性型のプラスミンとなる(**図1**)。プラスミンは，血栓の主成分であるフィブリンを分解することにより血栓を溶解する。哺乳類のプラスミノーゲンアクチベーターは，ウロキナーゼ型のプラスミノーゲンアクチベーターと組織型のプラスミノーゲンアクチベーターに大別される。前者は古くは尿中に見出されたことから，現在でも単にウロキナーゼと呼ばれる。

ウロキナーゼは，セリンプロテアーゼの1つで，411個のアミノ酸残基からなる糖タンパク質である。ウロキナーゼは，プロウロキナーゼ(411個のアミノ酸残基からなる1本鎖糖タンパク質，分子量約55,000)として産生された後に，プラスミンやカリクレインによって158番目のリジンと159番目のイソロイシンの間が限定分解されることによって生じるA鎖(N末端側ペプチド，分子量約22,000)およびB鎖(C末端側ペプチド，分子量33,000)からなる2量体タンパク質である。ウロキナーゼは，活

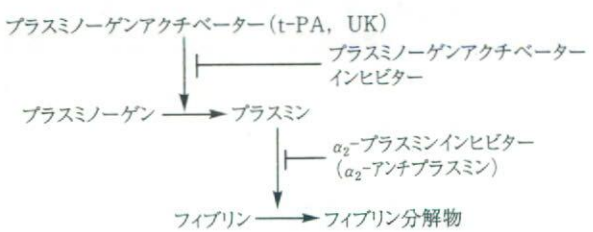


図1　血栓溶解機構
t-PA：組織プラスミノーゲンアクチベーター
UK：ウロキナーゼ

性型高分子量ウロキナーゼ(分子量約55,000)または，さらに分解された低分子量ウロキナーゼ(分子量約31,500)として存在する。

INNに収載されているウロキナーゼは，「A plasminogen activator isolated from human sources」とあるようにヒト由来である。わが国で承認され，日局に収載されているウロキナーゼは，ヒト尿から精製した高分子量型ウロキナーゼである。わが国では，急性心筋梗塞における冠動脈血栓の溶解剤として適用されている。ヒト尿由来ウロキナーゼは欧州でも承認されており，EPに収載されている。EP収載ウロキナーゼは高分子量および低分子量ウロキナーゼを含むものである。

米国で承認されているウロキナーゼは，尿由来ではなく，組織培養(新生児腎細胞)由来のもので，分子量約32,000の低分子量ウロキナーゼである。また，INNにはUrokinase Alfaが収載されており，これは遺伝子組換え型糖タンパク質で，非ヒト哺乳動物由来細胞で産生された高分子量ウロキナーゼである。

欧米で血栓溶解剤として使用されているストレプトキナーゼも，プラスミノーゲンアクチベーターである。ストレプトキナーゼは，黄色ブドウ球菌由来のタンパク質分解酵素で，プラスミノーゲンをプラスミンに活性化する。わが国では販売されていない。

(2)「-uplase」：ウロキナーゼ型プラスミノーゲンアクチベーター

「**-uplase**」はウロキナーゼ型プラスミノーゲンアクチベーター(urokinase-type plasminogen activator)に共通のサブステムで，JANにはNasar**uplase**(ナサルプラーゼ(細胞培養))が収載されている。

ナサルプラーゼは，ヒトプロウロキナーゼと同一のアミノ酸配列を持つ糖タンパク質である。ナサルプラーゼは，ヒト腎臓に由来する2倍体細胞の培養により繊維芽細胞をクローン化し，株化した細胞で産生される。急性心筋梗塞における冠動脈血栓の溶解を効能とした血栓溶解剤として承認されている。

ナサルプラーゼと同一のアミノ酸配列を持ち，糖鎖が異なる品目として，INNにNasar**uplase** Betaが収載されている。これは，マウス細胞で産生される遺伝子組換え糖タンパク質である。Nasaruplaseは，Alfaが収載されず，Betaが収載されている唯一の例である。その他，「**-uplase**」を持つ品目としてINNにはSaruplaseが収載されている。

(3)「-teplase」：組織プラスミノーゲンアクチベーター類

「**-teplase**」は，組織プラスミノーゲンアクチベーター(t-PA)類(tissue-type-plasminogen activator)に共通のサブステムである。t-PAは，ウロキナーゼと同様にプラスミノーゲンの560番目のアルギニンと561番目のバリンの間を切断することによって，プラスミノーゲンをプラスミンに活性化する(図1)。t-PAは，主に血管内皮細胞で産生される527個のアミノ酸残基からなる分子量約70,000の1本鎖糖タンパク質で，分子内の3カ所(Asn117，184，448)に糖鎖が結合している。N末端側から，フィンガードメイン，EGFドメイン，クリングル1ドメイン，クリングル2ドメイン，Catalyticドメインから構成されている(**図2**)。t-PAは，プラスミンによりCatalyticドメイン上の275番目のアルギニンと276番目のイソロイシンの間が切断されると，重鎖(N末端側)と軽鎖(C末端側)からなる2本鎖t-PAになる。2本鎖t-PAになると十分な活性を発揮するが，1本鎖でも2本鎖t-PAの約1/10の酵素活性を有する。t-PAがウロキナーゼと大きく異なる点は，フィブリン親和性を有する点である。t-PAは血栓に特異的に結合してプラスミノーゲンを活性化するので，血栓溶解効率が高い。これに対してウロキナーゼは，循環血液中のプラスミノーゲンを活性化するために，生じたプラスミンが特異的インヒビター(α_2-プラスミンインヒビター等)で失活したり，出血傾向を引き起こしたりする。t-PAの高いフィブリン親和性には，フィンガードメインとクリングル2ドメインが関与しているといわれている。

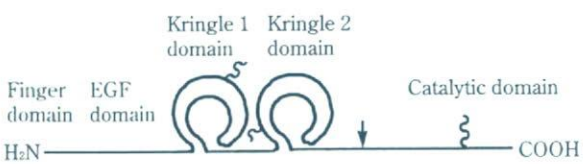
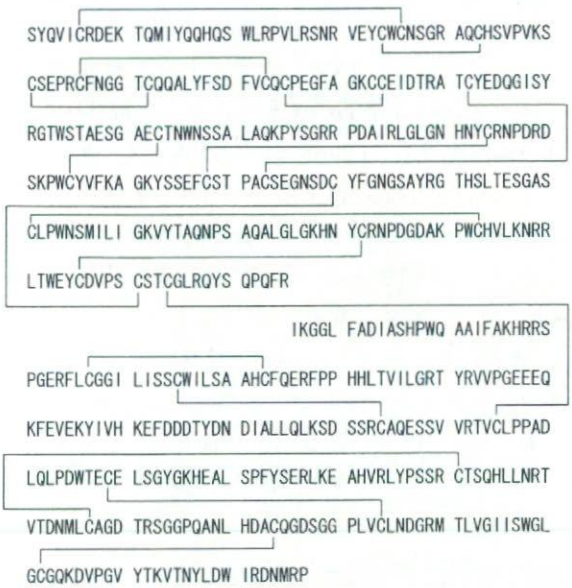


図2　t-PAの構造
フィンガードメイン：1～43番目
EGFドメイン：44～91番目
クリングル1ドメイン：92～173番目
クリングル2ドメイン：180～261番目
Catalyticドメイン：276～527番目
～～～：糖鎖
→：プラスミン限定分解部位

サブステム「**-teplase**」を持ちわが国で承認されている医薬品として，Al**teplase**(アル**テプラーゼ**)，Mon**teplase**(モン**テプラーゼ**)およびPami**teplase**(パミ**テプラーゼ**)がある。



N117，N184，N448：糖鎖結合

図3　アルテプラーゼの一次構造，ジスルフィド結合と糖鎖結合部位

アルテプラーゼは，遺伝子組換えヒト組織プラスミノーゲンアクチベーターで，CHO細胞によって産生される（図3）。虚血性脳血管障害急性期に伴う機能障害の改善（発症後3時間以内）および急性心筋梗塞における冠動脈血栓の溶解（発症6時間以内）に適用される。アルテプラーゼは，海外でも承認されており，EPおよびUSPにも収載されている。わが国では日局収載候補品目になっている。

t-PAは，血中からの消失が速く，静脈投与する場合は点滴投与が必要とされている。t-PAの肝臓での代謝には，クリングル1ドメイン上のAsn117に結合している高マンノース型糖鎖やEGFドメインが関与していると考えられている。そこで，血中での滞留時間を延長させるために，遺伝子工学的にt-PAを改変する研究が進められた。現在ではさまざまな改変型t-PAが血栓溶解薬として使用されている。

モンテプラーゼは，t-PAの84番目のシステインをセリンに変換した改変型t-PAで，ベビーハムスター腎細胞により産生される。わが国では急性心筋梗塞における冠動脈血栓の溶解（発症後6時間以内）および不安定な血行動態を伴う急性肺塞栓症における肺動脈血栓の溶解に適用されている。

パミテプラーゼは，t-PAのクリングル1ドメインを欠失させることによって血中半減期を延長し，かつt-PAが2本鎖に解離しないように天然型t-PAのN末端から275番目のアルギニンをグルタミン酸に変換してフィブリン親和性を回復させた改変型t-PAで，CHO細胞によって産生される。パミテプラーゼは，急性心筋梗塞における冠動脈血栓の溶解（発症後6時間以内）に適用されている。

サブステム「**-teplase**」を持つその他の品目としてJANには，Du**teplase**（デュ**テプラーゼ**），Lano**teplase**（ラノ**テプラーゼ**），Sil**teplase**（シル**テプラーゼ**），Na**teplase**（ナ**テプラーゼ**），およびEcol**teplase**（エコル**テプラーゼ**）が収載されている。欧米では，Tenec**teplase**とRe**teplase**が承認されているが，いずれもJAN未収載である。

Tenecteplaseは，CHO細胞で産生されるt-PA改変体で，103番目および117番目のアミノ酸残基がそれぞれアスパラギンおよびグルタミンに変換され，さらに296～299番目のアミノ酸残基がアラニンに変換されている。Reteplaseは，クリングル2ドメインとCatalyticドメインからなる改変体で，大腸菌で産生される糖鎖非結合タンパク質である。

その他サブステム「**-teplase**」を持つ医薬品として，INNにはAnis**treplase**およびDesmo**teplase**が収載されている。

「**-ase**」の項で述べたTisoki**nase**（チソキ**ナーゼ**）は，t-PAを示すサブステム「**-teplase**」を持たないが，天然型t-PAである。チソキナーゼは，527個のアミノ酸残基からなる糖タンパク質で，ヒト肺に由来する2倍体繊維芽細胞で産生される。血栓溶解剤として承認されている。

(4)「-diplase」：プラスミノーゲンアクチベーターと他の酵素との融合タンパク質

「**-diplase**」はプラスミノーゲンアクチベーターと他の酵素との融合タンパク質に与えられたサブステムである。サブステム「**-diplase**」を持つ品目として，Ame**diplase**がINNに収載されている。Ame**diplase**は，t-PAのクリングル2ドメインとプロウロキナーゼのC末端側ドメインから構成される遺伝子組換えキメラ型プラスミノーゲンアクチベーターである。

(5)「-lipase」：リパーゼ活性を持つ酵素

リパーゼ（lipase）活性を持つ酵素にはサブステム

「-lipase」が与えられている。リパーゼはグリセロールエステルを加水分解し，脂肪酸を遊離する酵素である。サブステム「-lipase」を持ちINNに収載されている品目として，遺伝子組換えヒト胆汁酸塩活性化リパーゼであるBucelipase Alfaや，*Rhizopus arrhizus*が産生するリパーゼRizolipaseがある。

(6)「-dismase」：スーパーオキシドジスムターゼ活性を持つ酵素

「-dismase」は，スーパーオキシドジスムターゼ(Superoxide dismutase)活性を持つ酵素に共通のサブステムである。スーパーオキシドジスムターゼは，異性化酵素(isomerase)の1種で，スーパーオキシドアニオンラジカルの不均化反応(下式)を触媒する。

$$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$$

スーパーオキシドジスムターゼは，細胞内に発生したスーパーオキシドアニオンラジカル濃度を低下させることにより，DNA，膜脂質，タンパク質，炭水化物の酸化的損傷を抑制し，酸素障害を防御している。サブステム「-dismase」を持ちINNに収載されている品目として，LedismaseとSudismaseがある。また，「-dismase」を持たないが，INNに収載されているOrgoteinは赤血球由来スーパーオキシドジスムターゼである。そのPEG化体PegorgoteinもINNに収載されている。

ステム「-ase」を持つその他の酵素性医薬品として，INNやJANには多くの糖分解酵素も収載されている。それらは本連載の第21回で紹介する予定である。

以上，今回は，酵素を示すステム「-ase」を持つ医薬品の中から，タンパク質分解酵素，ウロキナーゼ型プラスミノーゲンアクチベーター類，組織プラスミノーゲンアクチベーター類，プラスミノーゲンアクチベーターと他の酵素との融合タンパク質，リパーゼおよびスーパーオキシドジスムターゼ活性を持つ酵素を紹介した。

■参考文献

本稿作成に使用した参考文献は，本連載第5回(本誌2006年12月号)に記載している。また，以下の文献を参考にした。

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RESEARCH ARTICLE

A Human Phospholamban Promoter Polymorphism in Dilated Cardiomyopathy Alters Transcriptional Regulation by Glucocorticoids

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Communicated by Nancy B. Spinner

Depressed calcium handling by the sarcoplasmic reticulum (SR) Ca-ATPase and its regulator phospholamban (PLN) is a key characteristic of human and experimental heart failure. Accumulating evidence indicates that increases in the relative levels of PLN to Ca-ATPase in failing hearts and resulting inhibition of Ca sequestration during diastole, impairs contractility. Here, we identified a genetic variant in the PLN promoter region, which increases its expression and may serve as a genetic modifier in dilated cardiomyopathy (DCM). The variant AF177763.1:g.203A>C (at position –36bp relative to the PLN transcriptional start site) was found only in the heterozygous form in 1 out of 296 normal subjects and in 22 out of 381 cardiomyopathy patients (heart failure at age of 18–44 years, ejection fraction = 22 ± 9%). In vitro analysis, using luciferase as a reporter gene in rat neonatal cardiomyocytes, indicated that the PLN-variant increased activity by 24% compared to the wild type. Furthermore, the g.203A>C substitution altered the specific sequence of the steroid receptor for the glucocorticoid nuclear receptor (GR)/transcription factor in the PLN promoter, resulting in enhanced binding to the mutated DNA site. These findings suggest that the g.203A>C genetic variant in the human PLN promoter may contribute to depressed contractility and accelerate functional deterioration in heart failure. Hum Mutat 0,1–8, 2008. © 2008 Wiley-Liss, Inc.

KEY WORDS: promoter; polymorphism; transcriptional factor; GR; GRE; cardiomyopathy; PLN; SR Ca-ATPase

INTRODUCTION

Heart failure is a multifactorial syndrome in which intrinsic myocardial dysfunction contributes to cardiac dilation and diminished ejection performance, leading to progressive cardiac deterioration or sudden death [Richardson et al., 1996; Seidman and Seidman, 2001]. Genes causally associated with cardiomyopathy have been identified through nonbiased genetic analysis or by candidate gene studies in experimental system [Geisterfer-Lowrance et al., 1996; Franz et al., 2001]. Thus, molecular modifiers of heart failure include mutations of genes that encode cytoskeletal, sarcomeric, nuclear membrane, and calcium handling sarcoplasmic reticulum (SR) proteins. These findings have implicated pathogenic mechanisms whereby perturbation of structural integrity, contractile force dynamics, and calcium regulation within the cardiac myocyte intrinsically contribute to myocardial disease.

Abnormal calcium homeostasis is a prototypical mechanism for contractile dysfunction in failing cardiomyocytes. Depressed calcium cycling in experimental and human heart failure reflects, at least in part, impaired calcium sequestration by the SR [Chien, 2000; MacLennan and Kranias, 2003]. Calcium sequestration is mediated by a Ca-transport ATPase (SERCA2a), whose activity is modulated by alteration in the expression and phosphorylation of phospholamban (PLN; MIM# 172405) [Luo et al., 1996; Simmerman and Jones, 1998]. In experimental models, expression levels of PLN closely correlate with basal contractile parameters and their responses to β-agonists [Luo et al., 1994; Kadambi et al., 1996; Brittsan et al., 2000; Dash et al., 2001]. In human heart failure, the levels of PLN are increased relative to SERCA2a, resulting in higher inhibition of the Ca-pump's Ca-affinity, which impairs relaxation [Beuckelmann et al., 1992; Meyer et al., 1995; Hasenfuss, 1998]. As a double insult, the phosphorylation status of PLN is decreased, leading to increased inhibitory function and further depression of SR Ca-cycling. Thus, PLN is a major

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Ca-regulatory protein and efforts have concentrated on identifying naturally occurring mutations in the human PLN gene, which may perturb its activity and contribute to dilated cardiomyopathy (DCM). Indeed, three mutations in the coding region of the human PLN gene have been identified that are associated with familial cardiomyopathy [Haghighi et al., 2003, 2006; Schmitt et al., 2003]. However, parallel studies on genetic variants in the PLN promoter region, which may alter its expression levels, are limiting.

The PLN gene is located on human chromosome 6 [Fujii et al., 1991] and the 200 bp of its 5′ flanking region exhibits significant sequence homology between human, rabbit, rat, and mouse [Fujii et al., 1991; Haghighi et al., 1997; McTiernan et al., 1999a, 1999b]. Importantly, this segment of the 5′ upstream region of the human PLN gene contains conserved consensus motifs for GATA, CP1/NF-y, M-CAT, and E-box elements, which are also found in other mammalian species [Haghighi et al., 1997; McTiernan et al., 1999a]. However, the importance of these elements in regulation of PLN gene expression under physiological and pathophysiological conditions remains uninvestigated. Indeed, most studies indicate that cardiac PLN expression levels are maintained under stress and remodeling conditions, including pressure overload, hypertrophy, and failure [Ito et al., 2001; Kogler et al., 2003; Mills et al., 2006]. A recent study reported the presence of a rare human mutation in this highly conserved PLN promoter region (A>G at −77 bp), which was associated with increased PLN (1.5-fold) expression [Minamisawa et al., 2003]. This variant was found in 1 out of 87 hypertrophic cardiomyopathy patients, suggesting a role of the PLN promoter mutant in depressed Ca cycling, leading to hypertrophy.

In this study, we sought to identify naturally occurring PLN promoter mutations in nonfamilial heart failure patients. A novel point genetic variant (A>C) at position AF177763.1:g.203A>C (at −36bp relative to the PLN transcriptional start site: −36A>C) in the 5′ UTR region of the PLN gene was detected only in the heterozygous state in 22 heart failure patients and one normal subject. In vitro studies on the functional significance of this genetic variant revealed that it increases PLN expression levels by altering glucocorticoid nuclear receptor (GR)-mediated regulation of transcription.

MATERIALS AND METHODS

Mutation Identification

Informed consent was obtained from participating subjects. All protocols were approved by the institutional review board of the Onassis Cardiac Surgery Center (Athens, Greece) or the University of Cincinnati College of Medicine, (Cincinnati, OH). Genomic DNA was isolated either from whole blood or from paraffin blocks containing heart tissue. The genomic reference with GenBank accession number AF177763.1 was used to retrieve the PLN sequence corresponding to proximal promoter and exon 1. A 600-bp fragment of the PLN gene, containing the PLN promoter region was amplified by PCR, using 60 ng of genomic DNA and a high-fidelity *Taq* polymerase. The primers were: sense, 5′CTAAGCCTGAAGATGC3′ and antisense, 5′CCAGTAACCA GGATC3′, tagged with M13 forward and reverse primer sequences, respectively. The conditions were: one cycle at 94°C for 3 min, linked to 30 cycles at 94°C for 1 min, 47°C for 1 min, and 72°C for 1 min, followed by one cycle at 94°C for 1 min, 53°C for 1 min, and 72°C for 10 min. The gel purified PLN DNA fragment was sequenced using automated dye-primer chemistry. The generated sequences were compared with the reported human PLN sequences by a computational method and the electropherograms were inspected individually for confirmation. The GenBank accession number AF177763.1 was used as a reference for numbering the PLN promoter polymorphism.

Echocardiography

Comprehensive 2D and Doppler echocardiography was performed according to the recommendations of the American Society of Echocardiography [Levy et al., 1990]. Left ventricular dimensions (interventricular septum end-diastolic thickness [IVEDT], left ventricular posterior wall end-diastolic thickness [PWEDT], left ventricular end-systolic and end-diastolic diameter [LVESD and LVEDD]) were measured with M-mode echocardiography, using the left parasternal window. Left ventricular volumes and ejection fraction (LVEF) were determined by apical two- and four-chamber views using the modified Simpson rule [Levy et al., 1990].

Cloning of the Human PLN Gene Promoter-Reporter Constructs

A PCR-based strategy was employed using high-fidelity DNA polymerase to amplify the mutant region from human PLN genomic DNA, comprising the upstream PLN promoter. A 510-bp DNA fragment was PCR-amplified from normal and DCM genomic DNA utilizing the primers 5′-TACCTGTGTTTATTTTTCTC-3′ and 5′-AAGAAGAATTACCAAAGTCAGC3′. To facilitate cloning, Kpn I and Xho I sites were added to the beginning of the primers. The 510-bp fragment containing the engineered Kpn I and Xho I sites was subcloned into the pBlueScript vector (Stratagene, La Jolla, CA). The upstream PLN promoter region was verified by DNA sequence analysis. Then, the PLN promoter fragment containing either the nucleotide transition, −36A>C, or the wild type sequences of the PLN gene was digested with Sac I and Pst I, and cloned into pGL3-Basic (Promega, Madison, WI) to create the PLN promoter-luciferase reporter constructs.

About 600 bp of 5′ upstream of the PLN gene sequences were scanned for putative transcription factor binding sites, using public domain software (Transcription Element Search Software; www.cbil.upenn.edu/tess; TFBLAST of TRANSFAC 6.0; Biobase Corporation, Beverly, MA; www.gene-regulation.com/cgi-bin/pub/programs/tfblast/tfblast.cgi).

Cardiomyocyte Culture, Transient Transfection, and Luciferase Assays

Ventricular myocytes were isolated from 1-day-old Sprague-Dawley rats and cultured as described [Minamisawa et al., 2003]. For promoter-reporter studies, after 24 hr incubation with serum-free medium, the myocytes were transiently cotransfected with 300 ng of each PLN luciferase test plasmid and 75 ng of phRL-TK control plasmid (Promega). The cells were harvested in Passive Lysis Buffer (Promega) 48 hr after transfection, and were stored at −80°C until processed for the luciferase assay. The cells were allowed to grow in the absence or presence of 3 μM dexamethasone for the last 45 hr of the 48-hr incubation period. Luciferase assays were performed according to the protocol of the Dual Luciferase Assay System (Promega). Each data point represents the mean and the standard error of the mean (SEM) of seven experiments.

Electrophoretic Mobility Shift Assays

Nuclear extracts from ventricular tissue samples were prepared as described previously [Brown et al., 2005] with modifications. Briefly, ventricular tissue was pulverized at liquid N_2 temperatures, homogenized at low speed in buffer containing 10 mM HEPES

(pH 9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 25 μg/ml leupeptin, 0.2 mM sodium orthovanadate, and 0.1% (vol/vol) Triton X, then vortexed and incubated on ice for 10 min. After centrifugation (5,000 *g* for 10 min), the pellet was suspended in solution containing 20 mM HEPES (pH 7.9), 25% (vol/vol) glycerol, 0.6 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethanesulphonylfluoride (PMSF), 0.5 mM DTT, 25 μg/ml leupeptin, and 0.2 mM sodium orthovanadate, and then vortexed. This suspension was incubated on ice for 40 min with rigorous vortexing every 10 min. After centrifugation (10,000 *g* for 15 min), the supernatant was retained as a crude nuclear extract. Protein concentrations were determined using a Bio-Rad (Hercules, CA) protein assay with bovine serum albumin as a standard.

A double-stranded 20-bp oligodeoxynucleotide, containing the PLN promoter wild type (5′-CCTCCCTAG{A}{ACACTTTTTC-3′; underlined, glucocorticoid binding element) or mutant form (5′-CCTCCCTAG{C}{ACACTTTTTC-3′; bold, mutated nucleotide) was end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (Promega), and was purified using a G-50 Sephadex column (Amersham Pharmacia Biotech, Piscataway, NJ). The binding reactions were performed in a final volume of 10 μl that contained 20 μg of nuclear protein, 10 mM Tris · HCl (pH 7.5), 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol (vol/vol), and 1 μg of poly(dI-dC). After a 10-min preincubation at room temperature, the labeled probe (1×10^5 cpm/reaction) was added to each reaction and the reactions were incubated for an additional 20 min at room temperature. The DNA-protein complexes were separated on 6% nondenaturing polyacrylamide gels in 1 × Tris borate-EDTA buffer. Gels were vacuum-dried and exposed to X-ray film at –20°C, using intensifying screens. Competition assays with 100-fold molar excess of unlabeled consensus oligodeoxynucleotide or control nonspecific oligodeoxynucleotide were performed to ensure that the signal was specific. The commercially available oligonucleotide containing the common glucocorticoid consensus, 5′-GACGGTACAAAATGTTCTAGG-3′ (Active Motif, Carlsbad, CA) and antiglucocorticoid antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used for specific binding activity confirmation. A double-stranded 22-bp oligodeoxynucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) containing a consensus nuclear factor-κB (NF-κB) binding site (underlined) was used as positive control.

Statistics

Data are presented as mean ± SEM. Statistical analysis was performed using two-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test. A P value of < 0.05 was considered statistically significant. The agreement with the Hardy-Weinberg expectations (HWE) of genotype frequencies was determined using the chi-squared test based on the number of observed and expected heterozygotes and the exact test based on the number of observed and expected genotypes [Guo and Thompson, 1992].

RESULTS

Clinical History

A total of 381 DCM patients and 296 normal subjects without any known cardiomyopathy history were recruited from the University Hospital, Cincinnati Heart Failure/Transplant Program (Cincinnati, OH) and the Onassis Cardiac Surgery Center (Athens, Greece). The clinical characteristics and the demographic data for the DCM populations are summarized in Table 1.

TABLE 1. Clinical Characteristics of the United States and Greek DCM Patients With Heart Failure

	Ethnicity	
	United States	Greek
n	163	218
Age (years)	44.95 ± 3.3	40 ± 6.2
Gender (%)		
Male	72	82
Female	28	18
Etiology (%)		
Dilated cardiomyopathy	94.27	100
Ischemic cardiomyopathy	5.76	—
Functional class (% NYHA III/IV)	67.2	70.6
LVEF (%)	23.6 ± 8	26.4 ± 6

DCM, dilated cardiomyopathy; LVEF, left ventricular ejection fraction; NYHA, New York Heart Association Classification.

Comorbid conditions in the cohorts included: hypertension (8%), diabetes (6%), hypercholesterolemia (12%), and atrial fibrillation (12%). The medications used by the DCM patients were angiotensin-converting enzyme (ACE) inhibitors (97%), diuretics (94%), digoxin (98%), beta blockers (75%), Ca-channel blockers (12%), and antiarrhythmics (45%).

Identification of a Genetic Variant in the Human PLN Promoter Region

In the initial discovery study, the PLN gene promoter region 600 bp upstream from the transcriptional start site [McTiernan et al., 1999a] was sequenced in 40 unrelated Greek DCM patients. The sequencing of this region identified a single nucleotide transition from A>C at position AF177763.1:g.203A>C (at –36bp relative to the PLN transcriptional start site: –36A>C) (Fig. 1A). We subsequently screened an additional 178 Greek DCM patients to determine the frequency of this PLN genetic variant. The –36A>C substitution was found in another 15 patients (16/218 total) and it was only present in the heterozygous form, reflecting an allelic frequency of 3.66% in the Greek DCM population. To confirm the presence of this novel PLN promoter variant in a different heart failure population, 163 Caucasian DCM patients (University Hospital, Cincinnati Heart Failure/Transplant Program, University of Cincinnati, OH) were also screened. The –36A>C variant was found in the heterozygous form in six patients, reflecting an allelic frequency of 1.84%. The characteristics of the patients with the identified transition in the PLN gene in the two cohorts were similar (Table 2). The PLN –36A>C variant carriers presented with heart failure symptoms and were diagnosed with cardiomyopathy at ages ranging from 18 to 44 years. Echocardiography studies indicated severe left ventricular dilatation and systolic dysfunction (e.g., ejection fraction of 22 ± 9%). Their symptoms remained under control with drug treatments. However, some patients' symptoms progressively deteriorated (New York Heart Association [NYHA] Classification, NYHA class III), leading to the death of one patient at the age of 48 years and heart transplantation in another patient at the age of 46 years. The promoter variant –36A>C was found in only 1 normal control subject out of 296 screened. There were no departures from Hardy-Weinberg equilibrium for allelic frequencies in either DCM or control populations.

The promoter region of the human PLN gene, containing the genetic variant is a highly conserved region among species (Fig. 1B) [McTiernan et al., 1999a]. Therefore, it was hypothesized that this change in nucleotide sequence might alter PLN

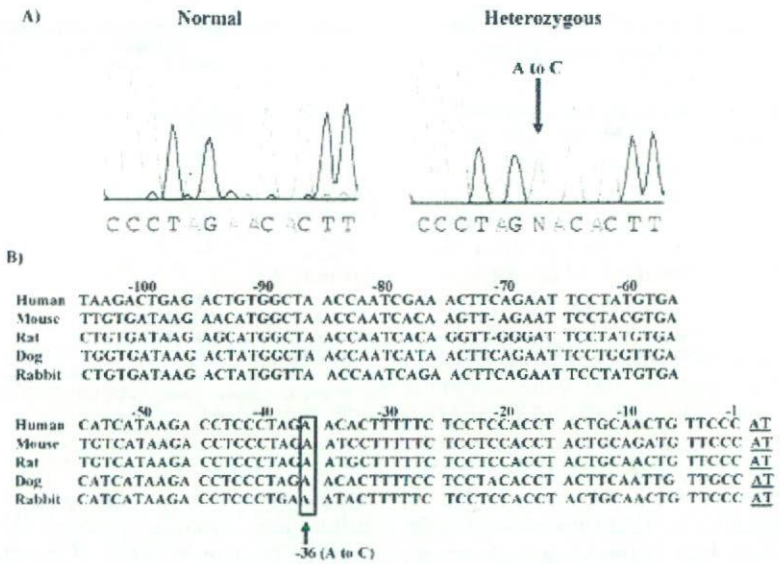

FIGURE 1. **Genomic DNA sequence analysis of the PLN promoter region. A: Partial nucleotide sequences of the PLN promoter region in normal subjects and DCM patients heterozygous for the AF177763.1:g.203A>C (at −36 bp from transcriptional start site: −36A>C) substitution. B: Sequence comparison of the proximal mammalian PLN promoter sequences was performed by the FASTA program (http://fasta.bioch.virginia.edu/fasta_www2/fasta_list2.shtml) (GenBank reference sequence numbers AF177763.1, AF037348.1, L03381.1, and M63600.1). The numbers correspond to human nucleotides upstream of exon 1 (transcription start site, underlined). The position of the A>C transition (boxed) is indicated. Gaps are shown by dash. Polymorphism numbering is based on using the GenBank accession number AF177763.1 for human PLN sequence corresponding to proximal promoter and exon 1 and the transcription start site as a reference. [Color figure can be viewed in the online issue, which is available at http://www.interscience.wiley.com.]**

TABLE 2. **Clinical Characteristics of Dilated Cardiomyopathy Patients With the −36A>C Substitution in the PLN Gene***

	Wild-type allele	Mutant allele
n	359	22
Age (years)	42 ± 3.1	40 ± 6
Etiology (%)		
Dilated cardiomyopathy	94.27	100
Functional class (% NYHA III/IV)	67.2	66.66
LVEF (%)	24.5 ± 8	22 ± 9

*Polymorphism numbering is based on using the GenBank accession number AF177763.1 for human PLN sequence, corresponding to proximal promoter and exon 1 as a reference.
DCM, dilated cardiomyopathy; LVEF, left ventricular ejection fraction; NYHA, New York Heart Association Classification.

promoter activity and consequently its regulation of SERCA2a, and thus contribute to the pathophysiology of heart failure.

In Vitro Assays of PLN Promoter Activity

To determine the potential functional importance of the identified genetic variation on PLN transcriptional regulation, we generated reporter constructs that expressed luciferase under the control of the putative promoter sequences from the human PLN gene. When neonatal rat cardiomyocytes were transiently transfected with luciferase reporters under the control of wild-type (PLN-WT) or "mutant" PLN (PLN-MT) promoters, the −36A>C transition resulted in a significant increase of 24% in transcriptional activity, compared to the wild-type promoter (Fig. 2A). To examine whether the −36A>C point transition in the PLN gene may alter regulation by any of the sequence-specific DNA-binding proteins, such as transcription factors, we performed a computer sequence search for putative regulatory binding sites. We identified a potential sequence for the glucocorticoid response element (GRE) within the mouse PLN promoter (Fig. 2B). Our DNA scanning revealed that the −36A>C substitution was within the putative glucocorticoid receptor binding site of the PLN promoter gene. To further investigate the regulation of PLN gene expression by the glucocorticoid response element, the luciferase reporter constructs of PLN-WT and PLN-MT were transiently transfected into rat neonatal cardiac cells in the absence or presence of dexamethasone. The induced luciferase activity of PLN-WT was significantly increased when dexamethasone was present, while there was no effect of dexamethasone on the PLN-MT, compared to basal levels (Fig. 2A). The lack of luciferase activity induction in PLN-MT following stimulation of transfected cells by dexamethasone may indicate that the genetic variant abolished the direct or indirect mediation of the dexamethasone-mediated enhancement of the reporter gene activity.

To further examine the functional significance of the −36A>C PLN promoter variant, gel mobility shift assays were employed. Using nuclear extracts from mouse heart, the binding assays showed that both synthetic WT (Fig. 3; lanes 13 and 14) and MT (Fig. 3; lanes 11 and 12) oligonucleotides were able to form a DNA-protein complex, indicating transcription factor binding. However, stronger binding was observed with the PLN-MT oligonucleotide, demonstrating that this sequence has a higher affinity for transcription factor binding. Binding was completely blocked in the presence of 100-fold excess of the cold-labeled WT (Fig. 3; lanes 3 and 4) or MT oligonucleotide (Fig. 3; lanes 5 and 6), used as specific competitors. Nuclear lysate was used as a negative control and it did not form any complexes in the presence of either synthetic WT or MT oligonucleotide (Fig. 3; lanes 7–10). However, an oligonucleotide containing a consensus NF-kB binding site, used as a positive control for nuclear lysate activity, yielded DNA-protein complexes in the lysates. These findings

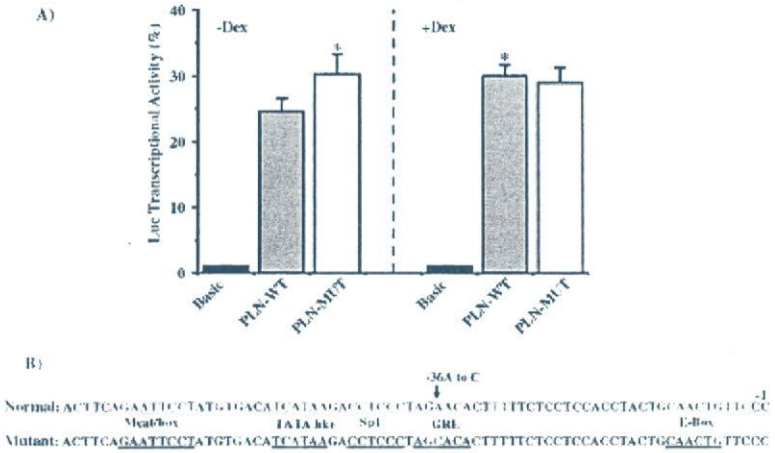

FIGURE 2. **Effect of the −36A > C genetic variant on human PLN promoter activity. A:** Rat neonatal cardiomyocytes were transiently transfected with a luciferase expression vector driven by PLN-WT or PLN-MT (−36A > C) promoters, and were cultured in the absence (left) or presence (right) of 3 μM dexamethasone (Dex) for 45 hr. Transcriptional activity of the promoters was defined as a ratio of firefly luciferase activity to Renilla luciferase activity in the same cells, and normalized to the mean basal transcriptional activity of the promoter-less pGL3-basic vector. **B:** Sequence alignment of the normal and mutant human PLN upstream promoter regions. The relative positions of the promoter starting site (-1) and of the potential regulatory sequences (underlined) are indicated. The values are expressed as means ± SEM (n = 7). $^*P < 0.05$ vs. PLN-WT without Dex (two-way ANOVA and Student-Neuman-Keuls test). Polymorphism numbering is based on using the GenBank accession number AF177763.1 for human PLN sequence corresponding to proximal promoter and exon 1 as a reference.

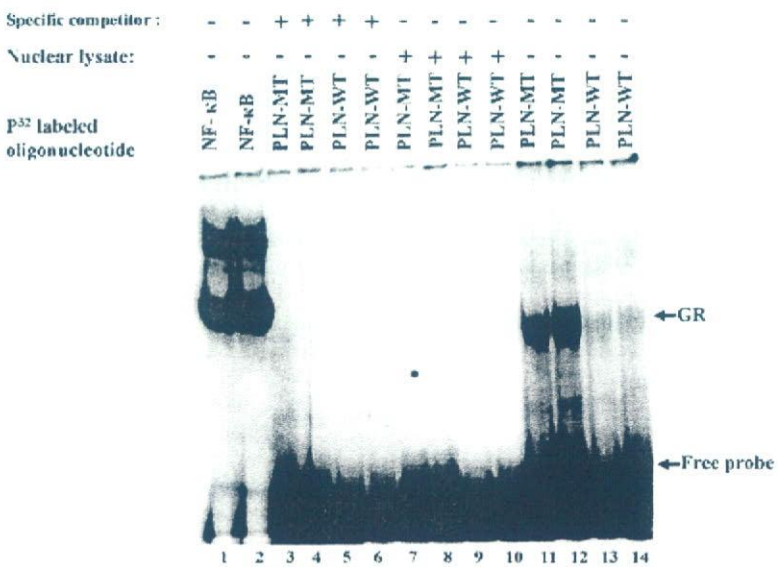

FIGURE 3. **Electrophoretic mobility gel shift assay of wild-type and genetically-altered glucocorticoid elements in the PLN promoter sequences. Electrophoretic mobility gel shift assays were used to determine DNA–protein complex formation using nuclear extracts from mouse hearts. NF-kB was used as a positive technical control (lanes 1 and 2); nonlabeled wild-type (PLN-WT, lanes 3 and 4) and altered (PLN-MT, lanes 5 and 6) were used as specific competitors; and nuclear lysate as a negative control (lanes 7–10); PLN-MT oligonucleotide (lanes 11 and 12) and PLN-WT oligonucleotide (lanes 13 and 14). Duplicate samples were assayed for each treatment.**

suggest that the quality of the nuclear lysates and the binding conditions were appropriate (Fig. 3; lanes 1 and 2).

To verify whether the binding activity from heart nuclear extracts reflects a specific interaction between the GR with the PLN-WT and PLN-MT probes, as predicted based upon computer searches, we employed a commercially available oligonucleotide, containing a known consensus GRE sequence. This oligonucleotide was used in DNA binding and competition studies, designed to assess specificity of our DNA-protein complexes. The GRE consensus oligonucleotide displayed a strong DNA-protein binding complex in nuclear extracts (Fig. 4; lane 3). Furthermore, this commercially available oligonucleotide could completely block DNA-protein complex formation with PLN-WT, PLN-MT, and the GRE oligonucleotide in the nuclear extracts (100-fold excess; Fig. 4; lanes 4–6). The consensus GRE containing oligonucleotide and the PLN promoter-derived sequences (PLN-WT and PLN-MT) demonstrated identical migration of the DNA-protein complexes (Figs. 3 and 4). It was interesting to note that the

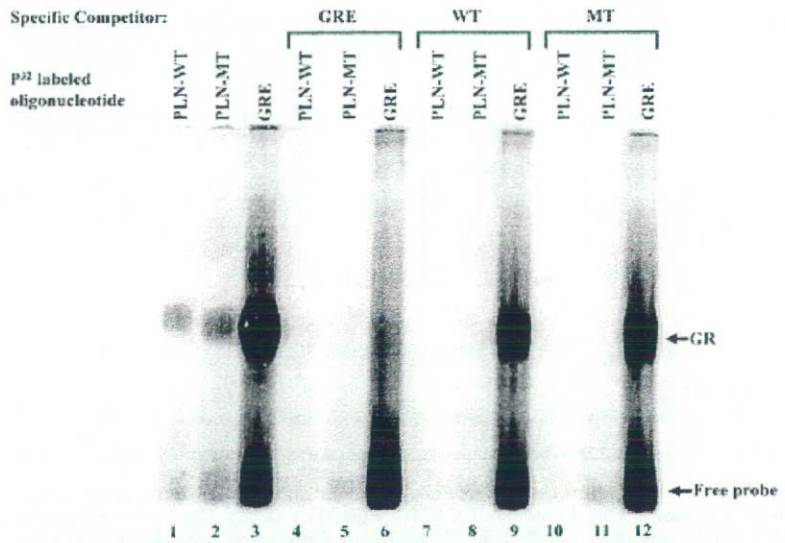

FIGURE 4. **Electrophoretic mobility gel shift assay of the PLN wild-type and genetically-altered glucocorticoid element specificity in the presence of common glucocorticoid consensus. Consensus glucocorticoid receptor element (GRE), PLN-WT, and PLN-MT motifs were used to examine binding specificity in cardiac nuclear extracts from wild-type mice. Lanes 1–3: cardiac nuclear extracts reacted with radiolabeled oligonucleotides of PLN-WT, PLN-MT, and consensus GRE sequences. Lanes 4–6: consensus GRE oligonucleotides were used as specific competitor (100 × unlabeled GRE oligonucleotides). Lanes 7–9 and lanes 10–12: PLN-WT and PLN-MT oligonucleotides were used as competitors (100 × -fold unlabeled oligonucleotide), respectively.**

PLN-WT or PLN-MT oligonucleotides could not completely compete with the common glucocorticoid consensus (Fig. 4; lanes 9 and 12). Taken together, these results indicate that the GR binds specifically to the PLN-WT and PLN-MT promoter sequences, albeit at lower affinity than the consensus GRE employed.

DISCUSSION

In this study, we identified a novel variant (–36A>C) in the human PLN promoter region in 22 heart failure patients and one normal subject, which appears to enhance promoter activity and alter the GR receptor binding element. Importantly, this PLN promoter variant was identified in two heart failure populations. The allelic frequencies in two ethnic populations and in controls were in Hardy-Weinberg equilibrium, indicating that this genetic variant is heritable and a combination of the –36A>C PLN variant with other genetic and environmental modifies may contribute to the time course of the disease in the patients. The identified nucleotide substitution is in close proximity to the putative TATA (5′-TCATAA-3′) boxes at position –48 to –53 in an evolutionarily conserved PLN gene region between species, and may play a significant role in regulating PLN gene expression. Indeed, in vitro studies of this genetic variant indicated that it may increase PLN expression levels and consequently, depress SR Ca cycling associated with cardiomyopathy. The functional significance of increased PLN levels in cardiac muscle has previously been demonstrated through the generation and characterization of transgenic mouse models [Kadambi et al., 1996; Dash et al., 2001]. Consistent with findings in transgenic mice, an increase in the apparent stoichiometry of PLN to SERCA2a, as a result of the PLN promoter genetic variant, may contribute to the depressed Ca cycling and deterioration of cardiac function.

Recently, there has been a considerable upsurge of interest in the influence of *cis*-acting genetic variations on gene transcription. Furthermore, these mutations and polymorphisms, found in various gene promoter regions, have been reported to affect gene expression and impact function [Collins et al., 2003; Hudson, 2003; Buckland et al., 2004; Guy et al., 2004; Schulz et al., 2006]. Importantly, the PLN promoter variant (A>C, underlined below), identified herein, was within a novel consensus sequence segment that matched a glucocorticoid receptor-binding site (5′-AGAA-GA-3′). Previous studies have shown that thyroid hormone and glucocorticoids regulate the expression of several genes, including calcium cycling proteins [Kiss et al., 1994, 1998; Smith and Smith, 1994; Brittsan et al., 1999; Muangmingsuk et al., 2000]. Thyroid hormone was reported to mediate changes in PLN protein levels [Kiss et al., 1994, 1998; Brittsan et al., 1999] possibly through interaction with thyroid hormone elements residing in the PLN promoter region. Glucocorticoids downregulate Na-Ca exchanger mRNA levels and activity in aortic myocytes [Smith and Smith, 1994], while they increase expression of alpha-myosin heavy chain (MHC) and decreased expression of beta-MHC in neonatal rat cardiomyocytes [Muangmingsuk et al., 2000]. These changes suggest that, similar to thyroid hormone-mediated transcriptional activation, the glucocorticoid effects may also be mediated in part through transcriptional mechanisms. Indeed, the level of PLN transcripts was significantly decreased, when rat neonatal cardiomyocytes were treated with cytokines (interleukin [IL]-1β, tumor necrosis factor [TNF]) [McTiernan et al., 1997], while dexamethasone significantly elevated the levels of PLN transcripts [McTiernan et al., 1997], indicating the direct effects of dexamethasone on PLN gene regulation. In this report, similar results were obtained with dexamethasone induction of PLN-WT promoter expression. In contrast, dexamethasone did not increase the luciferase transcriptional activity of the PLN-MT promoter, suggesting that the –36A>C substitution may have abolished the interaction site for glucocorticoid receptor elements in the PLN gene.

The role of transacting elements in the transcriptional activity of the PLN gene remains poorly understood and the nuclear proteins involved in the regulation of the gene through binding to these elements are unknown. Our previous studies on

characterization of the mouse PLN promoter indicated that 200 bp proximal to the transcriptional initiation site is sufficient for moderate (40%) expression of PLN levels [Haghighi et al., 1997]. The dexamethasone-responsive PLN gene sequences are located within the 200-bp proximal promoter region of the mouse and human PLN gene, which are highly conserved between species [Haghighi et al., 1997; McTiernan et al., 1999a]. Increased luciferase activity in the promoter-reporter studies suggest that GREs within this region may contribute to the modulation of transcriptional regulation via DNA–protein interactions of the PLN gene as further supported by electrophoretic mobility gel shift assay studies. Obviously, the limitation of this study is that the upregulation of the PLN promoter activity presented here is primarily from in vitro studies; in vivo relevance of these finding could not be performed due to lack of cardiac biopsies from affected individuals.

The glucocorticoid receptor is a ligand-dependent transcription factor with both hormone and DNA binding domains, affecting the transcription of specific genes [Schoneveld et al., 2004]. Specifically, glucocorticoid hormones are the major mediators of systemic stress responses [Brent et al., 1991] and it has been suggested that they may regulate SR function and cellular calcium homeostasis in the myocardium [Rao et al., 2001; Aoyama et al., 2005]. The possible mechanisms may involve modulation of PLN phosphorylation through Ca/calmodulin-dependent protein kinase II (CaM kinase II) [Rao et al., 2001]. Interestingly, the CaM kinase II dependent phosphorylation site of PLN, Thr17, has been implicated in stress responses of the cardiomyocytes [Hagemann et al., 2000; Zhao et al., 2004]. Therefore, under stress conditions, GR modulation of PLN activity and/or expression levels may influence SR Ca cycling and myocardial function, which may be beneficial during early cardiac remodeling but deleterious under pathophysiological conditions. However, the abolished PLN GRE site by the −36A > C genetic variant eliminates the GR-mediated regulation, resulting in chronic increases in PLN expression levels and inhibition of SERCA activity, which may accelerate deterioration of function in DCM.

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細胞・組織加工医薬品の品質と安全性確保への提言

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昨年は，我が国初の細胞・組織加工医薬品となる培養皮膚製品の承認，ヒト（自己）由来細胞・組織加工医薬品等の品質及び安全性の確保に関する指針案の公表，そして，ヒトiPS細胞作製の成功など，再生医療関連の大きなトピックが相次いだ。我が国では現在，骨，軟骨，角膜，心筋，血管，神経などの再生に関する臨床研究が精力的に行われている。また，樹状細胞や活性化リンパ球を用いたがん治療など細胞治療の臨床研究も活発である。これら臨床研究の成果が標準治療として広く一般に提供されていくためには，細胞・組織を含む製品を薬事法の規制下に医薬品として開発していくことにより，その品質・有効性・安全性を確保することが望まれる。本稿では，細胞・組織加工医薬品について，品質・安全性確保のための一般的な考え方と関連する規制状況等について海外での状況も含めて紹介する。

1　細胞・組織加工医薬品とは

細胞・組織加工医薬品等とは，ヒトあるいは動物由来の細胞・組織を加工して製造される医薬品又は医療用具と定義される。従って，細胞・組織加工医薬品は生きている細胞という刻一刻と変化する特性と，細胞という高度な有機体であるという特性を併せ持つことから，品質・安全性・有効性評価においてはこのような特性を充分に理解しておくことが必要となる。

細胞・組織の「加工」とは，疾患の治療や組織の修復又は再建を目的として，細胞・組織の人為的な増殖，細胞・組織の活性化などを目的とした薬剤処理，生物学的特性改変，非細胞・組織成分との組み合わせ，遺伝子工学的改変等を施すことを言う。したがって，いわゆる再生医療に用いられる細胞，例えば，軟骨細胞，血管内皮前駆細胞，間葉系幹細胞，筋芽細胞等の他に，免疫療法などで用いられる細胞やex vivo遺伝子治療用の細胞等も細胞・組織等加工医薬品の範囲に含まれる。加工を経ずに移植に用いられる角膜，造血幹細胞などは細胞・組織加工医薬品には含まれず，移植医療と位置づけられている。我が国と異なり，欧米ではこのような移植医療も含めて細胞治療として品質・安全性・有効性確保の対策をとっており，例えばフランスでは，1996年以降，細胞治療の60％は造血幹細胞を用いたものといわれている。

2　細胞・組織加工医薬品の品質と安全性確保 ― 規制環境の整備

細胞・組織加工医薬品に関して，その開発を適正に推進することを目的に，品質・安全性確保に関する規制関連文書が各極で出されている（表1，2）。いずれにおいても，細胞・組織加工医薬品では医薬品としての性質や患者への適用方法が製品ごとに大きく異なり多様であるため，画一的な規制を行うのではなく，目的とする製品の特性に応じてケースバイケースで対応すべきであるという考えが基本にある。細胞・組織を利用した医薬品の品質・安全性確保に関するこれまでの国内通知等で最も重要なものは，平成12年に出された医薬発第1314号通知

―別添1「細胞・組織利用医薬品等の取扱い及び使用に関する基本的考え方」

－別添２「ヒト由来細胞・組織加工医薬品等の品質・安全性確保に関する指針」である[1]。

別添１には，細胞・組織利用医薬品の製造にあたって，その採取行為から加工，製造における取り扱いや使用に際しての基本的要件が示されており，別添２には，ヒト由来細胞・組織加工医薬品について，その品質・安全性確保のために必要な基本的要件と，承認申請および治験前の「確認申請」に必要な資料の内容が示されている。

表１　細胞・組織加工医薬品に関連する指針や通知

◇ 細胞・組織を利用した医療用具又は医薬品の品質及び安全性の確保について
（医薬発第 906 号）
◇ 細胞・組織利用医薬品等の取扱い及び使用に関する基本的考え方
（医薬発第 1314 号　別添 1）
◇ ヒト由来細胞・組織加工医薬品等の品質及び安全性確保に関する指針
（医薬発第 1314 号　別添 2）
◇ 生物由来製品及び特定細胞由来製品の指定並びに生物由来原料基準の制定等について
（医薬発第 052001 号）
◇ 生物由来製品に関する感染症定期報告制度について
（医薬発第 051508 号）

表２　細胞・組織加工医薬品に関する海外の指針や通知

◇ Guidance for Industry : Guidance for Human Somatic Cell Therapy and Gene Therapy. FDA/CBER, 1998
◇ Suitability Determination for Donors of Human Cellular and Tissue-based Products. FDA/CBER, 1999
◇ Guidance for Reviewers : Instructions and Template for Chemistry, Manufacturing, and Control (CMC) Reviewers of Human Somatic Cell Therapy Investigational New Drug Applications (INDs). (DRAFT) FDA/CBER, 2003
◇ Points to Consider on the Manufacture and Quality Control of Human Somatic Cell Therapy Medicinal Products. EMEA/CPMP/BWP, 2001
◇ Guideline on Human Cell-based Medicinal Product (DRAFT) EMEA/CHMP, 2007

「確認申請」とは，治験計画の届出を行う前に，厚生大臣に当該治験薬の安全性及び品質の確認を求める申請のことであり，細胞・組織加工医薬品と遺伝子治療薬について義務化されているものである。確認申請の目的は，当該医薬品等の治験を開始するに当たって支障となる品質及び安全性の問題が存在するか否かを確認することであり，未知・未経験の要素が多い細胞・組織加工医薬品の開発を安全に行うために設けられている制度であると言える。

一方，基本的考え方および指針が示されて７年余りが経過したが，これまでの細胞・組織加工医薬品の開発状況を見ると，次々に製品が市場に提供されるという状況ではなく，確認申請の段階でどの程度のデータが必要とされるのかが分かりにくいという指摘や，確認申請がなされてから承認までの期間が長いという指摘もあった。そこで，その後の科学的進歩や経験の蓄積に基づき，細胞・組織加工医薬品の適正な開発を推進するという立場から現在，第 1314 号通知の見直し作業が進められている。改訂後の指針はヒト自己由来細胞製品とヒト同種（他家）由来細胞製品に分けて作成されることとなっており，平成 19 年にはヒト自己由来細胞製品を対象に第 1314 号通知の改訂案として，

－「ヒト（自己）由来細胞・組織加工医薬品等の製造管理・品質管理の考え方（案）」

－「ヒト（自己）由来細胞・組織加工医薬品等の品質及び安全性の確保に関する指針（案）」

が公表され，一般からの意見公募が行われた。

これらの製品の開発において，品質・安全性確保に必要なデータは非臨床試験のみならず臨床試験を通じて蓄積されていくという事実を踏まえ，改訂案では，確認申請の段階でどのようなデータが必要とされるか，すなわち治験を開始するために必要とされる要件にはどのようなものがあるかが改訂前より明確に示されている。また Q&A として，必要な背景説明もなされている。以下に，「ヒト（自己）由来細胞・組織加工医薬品等の品質及び安全性の確保に関する指針（案）」（表 3）の記載内容を中心に，細胞・組織加工医薬品の品質及び安全性確保のための方策について述べる。