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Crystal structure of troponin and the molecular mechanism of muscle regulation

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Abstract Troponin plays a central role in the regulation of skeletal and cardiac muscle contraction. The protein consists of three polypeptide chains (TnT, TnI and TnC) and is located on polymerized actin together with tropomyosin, forming muscle thin filament. We have determined the molecular structures of the core domains (relative molecular mass of 46 000 and 52 000) of human cardiac troponin in the Ca^{2+}-saturated form by X-ray crystallography. Analysis of the four structures derived from the two crystal forms reveals that the core domain is further divided into sub-domains, connected by linkers, making the entire molecule highly flexible. The structures of the troponin ternary complex suggests that the Ca^{2+}-binding to the regulatory TnC site displaces the carboxyl-terminal portion of TnI from actin/tropomyosin, thereby altering mobility and/or flexibility of the troponin/tropomyosin strand on the actin filament. These Ca^{2+}-dependent changes in the properties of the tropomyosin strand on the actin filament may in turn alter accessibility of myosin heads (motor protein) to the actin filament.

Keywords troponin, muscle regulation, x-ray crystallography, calcium, EF-hand, synchrotron

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Introduction

Muscle contraction is caused by interactions between two major contractile proteins, myosin and actin. In skeletal and cardiac muscle, either actin or myosin is polymerized to form filaments, the thin filament and the thick filament, respectively. The sliding action of these two filaments, which results in muscular contraction, is regulated by the intracellular Ca^{2+} concentrations. The molecular basis for Ca^{2+} regulation was initially established by Ebashi and co-workers in the 1960s, who identified troponin and tropomyosin as the principal proteins involved in this process [1]. Troponin, with a relative molecular mass of approximately 80 000 (80 kDa), is composed of three polypeptide chains, troponin T (TnT), troponin I (TnI) and troponin C (TnC) and is generally located along polymerized actin together with tropomyosin to form the backbone of the filament at a troponin : tropomyosin : actin ratio of 1 : 1 : 7 [2–5] (Fig. 1). Each subunit of the troponin complex performs a distinct function. TnT is the tropomyosin-binding subunit, TnI is the inhibitory subunit that blocks acto-myosin interactions and TnC is the Ca^{2+}-binding subunit that prevents TnI inhibition upon Ca^{2+} binding to its regulatory sites. The crystal structure of TnC reveals a dumbbell shaped structure with two globular domains connected by a central helix [6,7] (Fig. 2a). Each globular domain contains a pair of Ca^{2+}-binding sites. These sites in the N-terminal domain are specific for Ca^{2+} and responsible for regulation, while the C-terminal sites have a structural role and display higher affinity for Ca^{2+}. Moreover, the C-terminal sites are permanently occupied with Ca^{2+} or Mg^{2+} in the muscle cell. The crystal structure of TnC in complex with the N-terminal TnI peptide provides an insight into the mechanism of Ca^{2+} regulation [8]. The TnI fragment (residues 1–47) forms an amphiphilic α-helix that binds the hydrophobic cleft of the carboxyl-terminal domain of TnC (C-lobe) (Fig. 2b). Similar interaction in the amino-terminal domain (N-lobe) was proposed (Fig. 2c) on the

basis of this structure together with that of Ca^{2+}-saturated skeletal TnC in solution [9]. At a low Ca^{2+} concentration, the inhibitory region (IR, residues 137–148 in human cardiac TnI) interacts with actin, thereby blocking actomyosin ATPase activity [10–12]. Following an increase in Ca^{2+} concentration in the sarcoplasm, Ca^{2+} binding to the N-lobe facilitates accessibility of the hydrophobic patch for binding

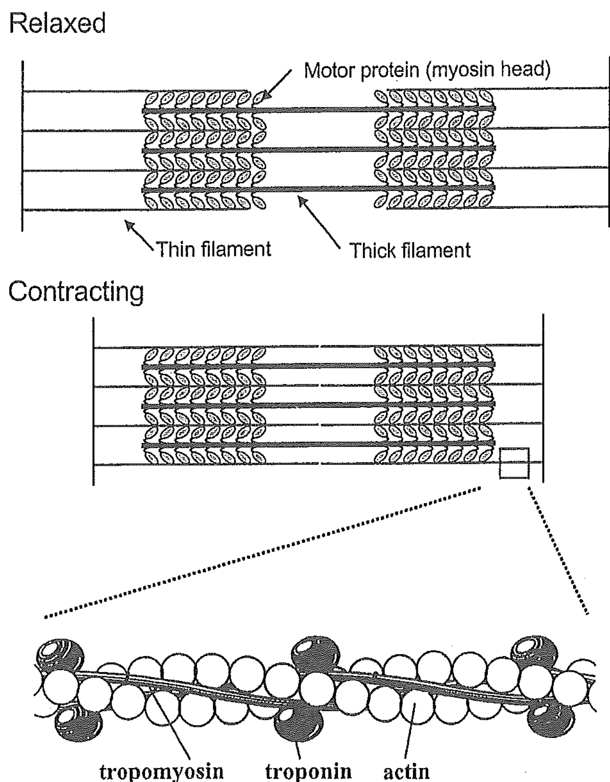

Fig. 1 Schematic representation of the ultrastructure of striated muscle.

to the second amphiphilic α-helix of TnI, which is located immediately downstream of the inhibitory region. The inhibitory region detaches form actin/tropomyosin, resulting in the prevention of inhibition. Direct interactions between the N-lobe of TnC and the TnI amphiphilic segment have been confirmed by NMR [13]. Although there is little doubt that the alternation in the interactions between TnC and TnI reported here have a primary role in triggering the initiation of muscle contraction, the mechanism by which troponin regulates the interactions between myosin heads and the non-equivalent seven actin monomers remains elusive. Troponin is believed to be anchored in the thin filament mainly through TnT. Thus, elucidation of the ternary complex (TnT/TnI/TnC) structure is crucial to determine the molecular mechanism of Ca^{2+} regulation. Here we report the first crystal structures of the troponin ternary complex. Our data show that the three polypeptide chains are folded around each other within the troponin molecule, and provide an insight into the mechanism by which the signal of Ca^{2+} binding to the regulatory TnC site is transmitted to other thin filament proteins. Details of the crystallographic structure solution are discussed in a recent publication by our group [14]. In this manuscript, we review the main findings derived from the structures and discuss its possible contribution of Ca^{2+} to the regulatory mechanism of muscle contraction.

Methods

Preparation

We crystallized two distinct preparations of the troponin core domain reconstituted from *Escherichia coli*-expressed recombinant human cardiac troponin subunits. One core domain consisting of TnC (residues, 1–161), TnI (residues, 31–163)

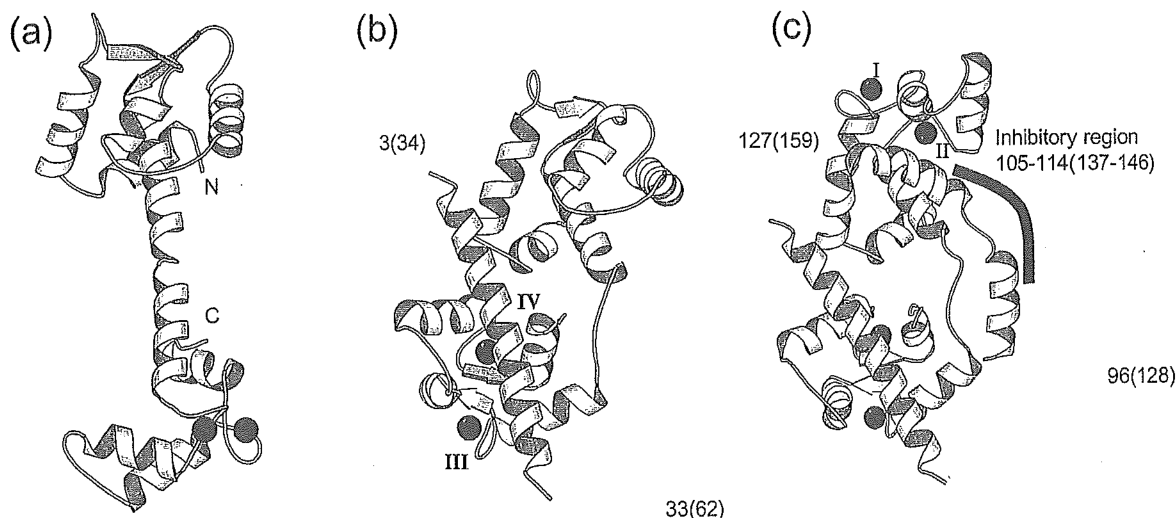

Fig. 2 Structures of TnCs. Crystal structure of TnC alone (a), TnC in complex with the N-terminal fragment of TnI (b), and a hypothetical model of TnC complexed with two putative TnC-binding segments of TnI (c). Amino acid numbers of the terminal residues of rabbit skeletal TnI segments and human cardiac TnI segments (in parentheses) are indicated. Bound Ca^{2+} ions (I–IV) are represented by black spheres.

and TnT (residues, 183–288) had a relative molecular mass of 46 K (Tn46K) and the other comprising TnC (1–161), TnI (31–210) and TnT (183–288) was 52 K (Tn52K). These core domains were equivalent to the chymotryptic fragments of rabbit skeletal Tn, which partially retain regulatory activity [15]. For TnI and TnC, cysteine-less variants (TnC (C35S/C84S) and TnI (C80A/C97)) were used to avoid the formation of the intermolecular disulfide bonds that prevent crystallization. Tn subunits were expressed and purified separately and dissolved in solution containing 6 M Urea. The mixture was dialyzed consecutively against NaCl solutions of 1, 0.5, 0.3 and 0.1 M, each containing 20 mM Tris/HCl at pH 8.0. Following refolding, the ternary complex was isolated by anion exchange chromatography and subjected to crystallization.

Crystallization

Native crystals were grown using the hanging-drop vapor diffusion technique with reservoir solution containing 20% polyethylene glycol (PEG) 3350, 15% glycerol, 0.1 M LiCl, 50 mM Tris/HCl and 5 mM $CaCl_2$, pH 8.0. Drops composed of 2 μl protein (10 mg ml^{-1}) and 2 μl reservoir solution were equilibrated with 0.5 ml reservoir solution for a minimum of two weeks at 20°C. Tn46K and Tn52K crystallized in space group $P2_1$ with unit cell dimensions of $a = 42.3$ Å, $b = 167.9$ Å, $c = 69.7$ Å, $\beta = 101.4°$, and $a = 48.3$ Å, $b = 169.5$ Å, $c = 68.5$ Å, $\beta = 102.4°$, respectively. Crystals were flash-frozen under nitrogen flow at 100 K after increasing the glycerol concentration 20% by soaking.

Structure determination

All diffraction data were collected at SPring-8 (the third generation synchrotron facility) in Japan (beam lines BL41XU, BL44B2 and BL45XU). Initial phases were obtained by the MAD (multiple anomalous dispersion) method [16] by using osmium and strontium derivatives of Tn46K crystals. The model was refined against the native Tn46K dataset to 2.6 Å resolution with a crystallographic R-factor of 26.4% (R-free = 29.8%). The Tn52K structure was solved by the molecular replacement method using the refined Tn46K model and refined to 3.3 Å resolution with a crystallographic R-factor of 25.1% (R-free = 30.8%). The asymmetric unit of each crystal form contained two troponin molecules. We resolved the structures of four molecules in total, designated Tn46KA, Tn46KB, Tn52KA and Tn52KB. Atomic coordinates and structure factors have been deposited in the Protein Data Bank under the accession codes 1J1D for Tn46K and 1D1E for Tn52K.

Results and discussion

Structure description

The overall architecture of the Tn52KB is depicted in Fig. 3a. Notably, the core domain of troponin is dominated by α-helices. To date, structures have been solved in the presence of Ca^{2+}. Thus, all three functional Ca^{2+}-binding sites (II, III and IV) are occupied (site I is not functional in cardiac TnC). The core domain is further subdivided into structurally distinct sub-domains, denoted the regulatory head and the IT-arm. The IT-arm has an asymmetric and elongated structure (~80 Å), which is mainly stabilized through two characteristic sets of interactions between the subunits. One comprises a parallel α-helical coiled-coil formed between the carboxyl-terminal portion of TnT (H2(T2)) and the middle portion of TnI (H2(I)). Each chain has 6.5 heptad repeats with hydrophobic residues at *a* and *d* positions, which are highly conserved among species [17]. The other involves hydrophobic interactions formed between the amino-terminal amphiphilic α-helix H1(I) of TnI and the hydrophobic cleft of the carboxyl-terminal domain (C-lobe) of TnC. As specified in Section 2, the metal binding sites in the C-lobe are permanently occupied by metal ions, irrespective of sarcoplasmic Ca^{2+} concentrations. Here, helix H2(T2) interacts with TnI by forming a coiled-coil on one side. The other side of the helix directly interacts with the Ca^{2+} binding loops of TnC C-lobe. Since the C-lobe is rigidly integrated into the IT-arm, this structure may remain unchanged irrespective of the physiological state of the thin filament. On the other hand, the regulatory head consists of the amino-terminal lobe of TnC (N-lobe) and the bound amphiphilic α-helix of TnI (H3(I)). As suggested previously [8,13], the amphiphilic H3(I) α-helix binds specifically to a conserved hydrophobic patch of the Ca^{2+}-saturated N-lobe of TnC in a Ca^{2+}-dependent manner. The H3(I) segment is located between the two putative actin/tropomyosin-binding sites, the inhibitory region and the carboxyl-terminus of TnI (C-TnI), which are both essential for inhibitory binding of TnI at a low sarcoplasmic Ca^{2+} concentration [12,18]. The structure of each sub-domain is almost identical in all four molecules, indicating that each sub-domain behaves as an individual unit. However the relative orientation between the two sub-domains is fairly variable (Fig. 3b). The largest differences are observed between Tn46KA and Tn46KB, where the IT-arm is rotated by 20° relative to the regulatory head, leading to a 27 Å displacement at the distal end of the IT-arm. Direct contacts between the IT-arm and the regulatory head are mediated by a small number of amino acid residues that are poorly conserved among the species. Therefore, the contacts are eventually stabilized by crystal-packing. Moreover, covalent links between the sub-domains involving the linkers connecting the D- and E-helices of TnC and the inhibitory region of TnI (between H2(I) and H3(I)) are not well defined in electron density maps suggesting that these linkers work as a universal joint. These findings collectively suggest that troponin molecule adopts multiple conformations with variable sub-domain orientations both on the muscle filament and within the crystal. Since troponin is fairly flexible, the molecule evades packing in the crystalline lattice for a long time.

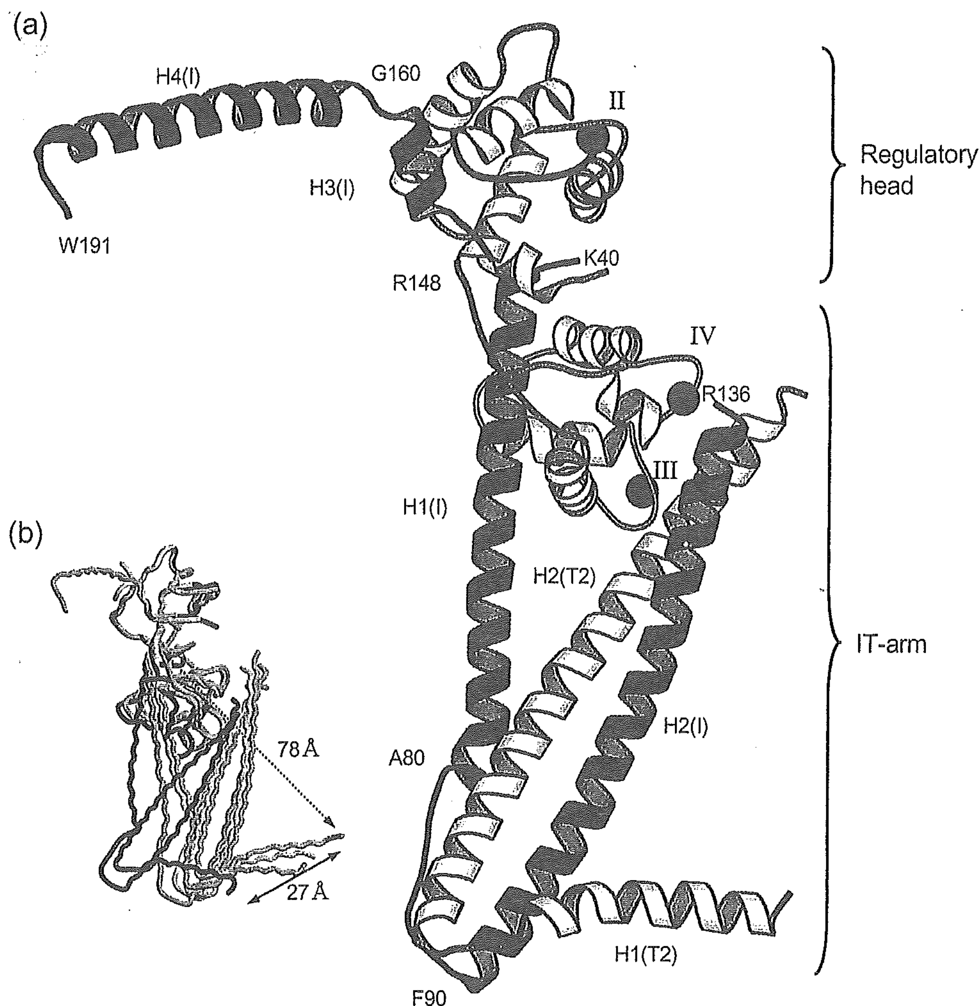

Fig. 3 Crystal structures of the core domain of human cardiac troponin. Ribbon representation of the Tn52KB molecule structure (a), and the superimposition of the four molecule structures with the best superimposition (least-square fit) of the regulatory head (b). The three bound Ca^{2+} ions (II–IV) are represented by black spheres.

Interactions between troponin and other thin filament components

Troponin is anchored to the thin filament mainly through tropomyosin binding to two distinct portions of TnT, specifically the amino-terminus (TnT1, residues 1–182) and the carboxyl-terminus (C-TnT, residues 272–288), irrespective of the sarcoplasmic Ca^{2+} concentrations (Fig. 4). The three-dimensional structure of TnT1 is currently unknown. However, a number of reports show that TnT1 has high α-helical content and is less susceptible to proteolysis, indicating that it forms a separate structural domain. On the basis of the present structures, the separation between TnT1 and C-TnT is estimated as 60 Å, although these two structural subdomains are not included in the current crystal structure. No part of the IT-arm has direct interactions with actin or tropomyosin. At low Ca^{2+} concentrations, the N-lobe of TnC dissociates from the H3(I) helix, resulting in another attachment of the carboxyl-terminal portion of TnI, denotes 'regulatory segment of TnI' (TnI_{reg}, residues 137–210) to the filament possibly to generate the ternary complex of actin/tropomyosin/TnI_{reg} [15].

Molecular mechanism of muscle regulation

During Ca^{2+} regulation, TnI_{reg} undergoes major changes, both with regard to position and conformation. At the high Ca^{2+} concentrations, TnI_{reg} associated with the N-lobe of TnC detaches from actin/tropomyosin as observed from the present crystal structure. On the other hand, at low Ca^{2+} concentrations, TnI_{reg} must form an extra attachment to actin/tropomyosin so that the troponin/tropomyosin strand is tied down to the actin filament (Fig. 4). The IT-arm may have an important role in the regulatory process. This subdomain is large, rigid and conserved between species and has no direct interactions with actin/tropomyosin. Moreover the location is interesting. The structure bridges two Ca^{2+}-independent attachments to actin/tropomyosin, specifically, TnT1 and C-TnT. The IT-arm resides immediately downstream of the tropomyosin anchoring site (C-TnT) and mobile TnI_{reg}. An intriguing possibility is that the formation of a third attachment by TnI_{reg} at low Ca^{2+} concentrations causes a minute rotation of the IT-arm about the pivotal point—the C-terminal end of the coiled-coil. The formation of the third attachment itself, as well as the rotation of the

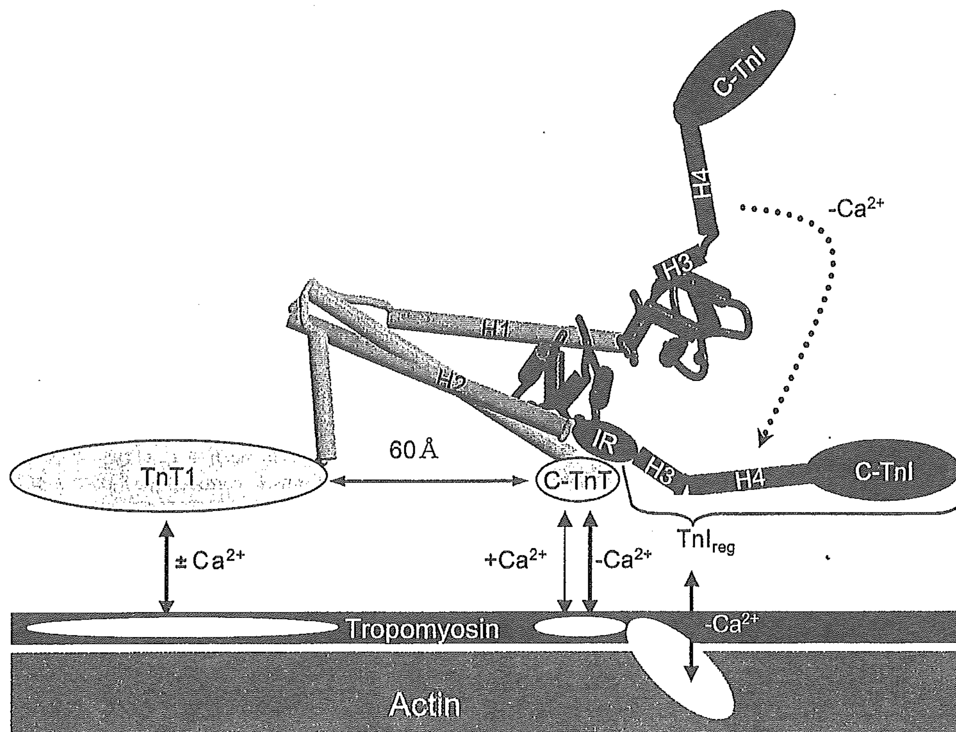

Fig. 4 Schematic representation of the interactions between troponin and other thin filament components. Potential actin/tropomyosin-binding portions, which are not included in the current structural model (TnT1, C-TnT, C-TnI and IR) are shown.

IT-arm, may alter the properties of the tropomyosin strand on the actin filament.

It is almost 30 years since the idea was put forward that tropomyosin acts, at least in part, by changing positions on actin, thus uncovering or modifying the myosin-binding site on actin when troponin binds Ca^{2+} (Fig. 5a). Although previous results are consistent with this steric blocking mechanism [19–21], the systematic fluorescence resonance energy transfer (FRET) experiment consistently failed to provide evidence of any major changes in the distance between tropomyosin and actin [22]. A more plausible explanation is that the strain imposed on the tropomyosin strand is altered. Consequently the mobility and/or flexibility of the tropomyosin strand is changed, which in turn modifies the accessibility of the myosin head to binding sites on the actin filament (Fig. 5b). Alternatively, it is possible that enhanced flexibility of the tropomyosin strand leads to conformational changes in actin subunits that induce higher flexural rigidity of the actin filament. The thin filament is more flexible in the presence of Ca^{2+} and its bending motion is accelerated by interactions of myosin heads with Mg^{2+}-ATP [23,24].

The nature of the changes in the thin filament remains to be elucidated. To determine the molecular mechanism of muscle regulation, it is crucial to clarify high-resolution structures of the whole thin filament complex (actin/tropomyosin/troponin) corresponding to each state during the contractile cycle [25]. Notably, thin filament-associated X-ray diffraction intensities [19–21] as well as electron micrographs [26] have been interpreted based on the assumption that the mass of the troponin/tropomyosin complex is distributed evenly and smoothly throughout the continuous 'tropomyosin strand'. With the present atomic structures, trials are currently underway to separately identify the mass of tropomyosin and that of troponin on the muscle thin filament.

Concluding remarks

The present study reveals that troponin can be divided into sub-domains, specifically, the regulatory head, the IT-arm, TnT1, C-TnT and TnI_{reg}. These sub-domains are connected by linkers, making the entire molecule fairly flexible. The crystal structures additionally provide an opportunity to visualize sites of genetic disorder in the troponin molecule that are associated with cardiac dysfunction [27]. In conjunction with previous results, data from the present structures enable us to outline a working hypothesis. Specifically, Ca^{2+} binding to the N-lobe of TnC induces interactions to the switch (H3(I) helix in TnI_{reg}) leading to the detachment of the entire TnI_{reg} from actin/tropomysin. This removal of TnI_{reg} releases the strain imposed on the tropomyosin strand via readjustment of the posture of the IT-arm. Changes in the properties of the tropomyosin strand may modify the accessibility of the myosin head to actin. Although this is the first hypothesis to explain the mechanism by which troponin works on the basis of its atomic structures, further work is necessary to establish complete understanding of the

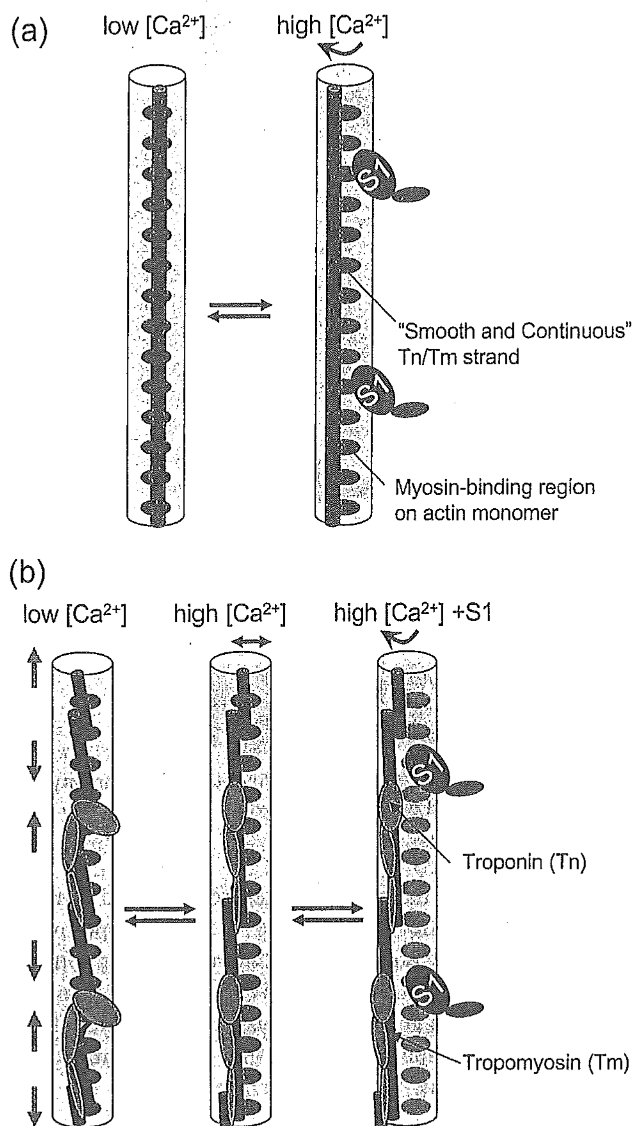

Fig. 5 The steric blocking model (a) and a model proposed in the present study (b). The steric blocking model assumes a smooth and continuous troponin/tropomyosin (Tn/Tm) strand. The new model assumes that the alternations in the strain imposed on the tropomyosin strand caused by the Ca^{2+}-dependent conformational changes in Tn, modify the accessibility of the myosin head to binding sites on the actin filament. Myosin heads (S1) are schematically drawn.

mechanism of muscle regulation. Ongoing efforts by our laboratory include solving the crystal structures of larger complexes, such as, Tn-Tm.

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Adrenomedullin: angiogenesis and gene therapy

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Nagaya, Noritoshi, Hidezo Mori, Shinsuke Murakami, Kenji Kangawa, and Soichiro Kitamura. Adrenomedullin: angiogenesis and gene therapy. *Am J Physiol Regul Integr Comp Physiol* 288: R1432–R1437, 2005; doi:10.1152/ajpregu.00662.2004.—Adrenomedullin (AM) is a potent, long-lasting vasodilator peptide that was originally isolated from human pheochromocytoma. AM signaling is of particular significance in endothelial cell biology since the peptide protects cells from apoptosis, promotes angiogenesis, and affects vascular tone and permeability. The angiogenic effect of AM is mediated by activation of Akt, mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2, and focal adhesion kinase in endothelial cells. Both AM and its receptor, calcitonin receptor-like receptor, are upregulated through a hypoxia-inducible factor-1-dependent pathway under hypoxic conditions. Thus AM signaling plays an important role in the regulation of angiogenesis in hypoxic conditions. Recently, we have developed a nonviral vector, gelatin. Positively charged gelatin holds negatively charged plasmid DNA in its lattice structure. DNA-gelatin complexes can delay gene degradation, leading to efficient gene transfer. Administration of AM DNA-gelatin complexes induces potent angiogenic effects in a rabbit model of hindlimb ischemia. Thus gelatin-mediated AM gene transfer may be a new therapeutic strategy for the treatment of tissue ischemia. Endothelial progenitor cells (EPCs) play an important role in endothelial regeneration. Interestingly, EPCs phagocytose ionically linked DNA-gelatin complexes in coculture, which allows nonviral gene transfer into EPCs. AM gene transfer into EPCs inhibits cell apoptosis and induces proliferation and migration, suggesting that AM gene transfer strengthens the therapeutic potential of EPCs. Intravenous administration of AM gene-modified EPCs regenerate pulmonary endothelium, resulting in improvement of pulmonary hypertension. These results suggest that in vivo and in vitro transfer of AM gene using gelatin may be applicable for intractable cardiovascular disease.

regeneration; endothelium; ischemia; pulmonary hypertension

ADRENOMEDULLIN (AM) IS A POTENT, long-lasting vasodilator peptide that was originally isolated from human pheochromocytoma (36). The peptide consists of 52 amino acids with an intramolecular disulfide bond, sharing slight homology with calcitonin gene-related peptide and amylin. Immunoreactive AM is detected in plasma and a variety of tissues including, blood vessels, heart, and lungs (19). Particularly, AM shows a variety of effects on the vasculature that include vasodilatation (23), regulation of permeability (16), inhibition of endothelial apoptosis (31), and promotion of angiogenesis (1, 35, 60). In addition, AM has protective effects against vascular injury, including oxidative stress (33, 69, 84). It is becoming clear that either activation or disruption of AM signaling might contribute to many pathological conditions, including hypertension (22), congestive heart failure (55), pulmonary hypertension (29), neoplastic growth (39), and inflammatory disease (59). To date, the major biological acitivities of AM in vitro and in vivo are *1)* vasodilation, *2)* diuresis and natriuresis, *3)* positive inotropic effect, *4)* inhibition of endothelial cell apoptosis, *5)* induction of angiogenesis, *6)* inhibition of cardiomyocyte apoptosis, *7)* suppression of aldosterone production, *8)* anti-inflammatory activity, and *9)* antioxidant activity. We and others have demonstrated that intravenous administration of AM decreases systemic and pulmonary arterial pressure and induces diuresis and natriuresis (47, 52, 65), suggesting that AM is involved in the regulation of vascular tone and body fluid. Subsequent studies have demonstrated beneficial hemodynamic effects and direct cardioprotective effects of AM infusion in the treatment of congestive heart failure (57, 61–64).

Until recently, only vascular endothelial growth factor (VEGF) (80), fibroblast growth factor (68), platelet-derived growth factor (37), and angiopoietin (74) were known to have profound angiogenic effects. More recently, however, the angiogenic potential of AM has attracted investigators' attention (35, 41, 59, 81). A previous study has shown that vascular abnormalities are present in homozygous AM knockout mice (70), suggesting that AM is essential for vascular morphogenesis. AM activates the PI3K/Akt-dependent pathway in vascular endothelial cells (58), which is considered to regulate multiple critical steps in angiogenesis, including endothelial cell survival, proliferation, migration, and capillary-like structure formation (27). These findings raise the possibility that AM plays a role in modulating angiogenesis and neovascular-

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ization. This review focused on the angiogenic effects of AM and the therapeutic potential of AM gene transfer for the treatment of intractable cardiovascular disease.

ENDOGENOUS AM PRODUCTION IN ISCHEMIC CONDITIONS

Hypoxia (14, 53) and cytokine production (73) in ischemic heart disease or septic shock, as well as shear stress (7) in hypertension and heart failure induce AM secretion by vascular cells (Fig. 1). We have shown that plasma AM level is increased in patients with acute myocardial infarction (40, 49), peripheral arterial occlusive disease (75), and congestive heart failure (28, 55). Tissue levels of AM peptide and mRNA are also markedly increased in ischemic myocardium (18, 50) and failing heart (8, 56, 78, 82). These findings suggest that expression of AM is upregulated under tissue ischemia and inflammation, both of which are associated with neovascularization. An in vitro study has demonstrated that AM is upregulated through a hypoxia-inducible factor-1 (HIF-1)-dependent pathway under hypoxic conditions (14). Thus hypoxia/HIF-1 is one of the most potent regulators of AM production (Fig. 1). A recent study has demonstrated that heterozygous AM knockout mice [AM(+/−)] show significantly less blood flow recovery with less collateral capillary development than their wild-type mice (20). Administration of AM promotes blood flow recovery and capillary formation in AM(+/−) mice. These findings suggest that endogenous AM may play an important role in the regulation of angiogenesis under ischemic conditions. Considering the angiogenic potency of AM, increased endogenous AM represents a compensatory mechanism as an angiogenic factor promoting neovascularization under hypoxic conditions.

ANGIOGENIC EFFECTS OF AM AND ITS SIGNALING PATHWAY

AM signaling is of particular significance in endothelial cell biology since the peptide protects cells from apoptosis (31), promotes angiogenesis (35, 60), and affects vascular tone (23). Angiogenesis is a multistep process that involves migration and proliferation of endothelial cells, functional maturation of the newly assembled vessels, and remodeling of the extracellular matrix (26). Akt, mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase 1/2 (ERK1/2), and focal adhesion kinase (p125FAK) play an important role in angiogenesis in endothelial cells. Kim et al. (35) demonstrated that AM activated Akt, MAPK/ERK1/2, and p125FAK in human umbilical vein endothelial cells (HUVECs), and produced increases in their DNA synthesis and migration. AM induced tube formation in HUVECs, and its effect was inhibited by pretreatment with a phosphatidylinositol 3′-kinase (PI3K) inhibitor or mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK)1/2 inhibitor. These findings suggest that AM exerts angiogenic activities through activation of Akt, MAPK, and p125FAK in endothelial cells (Fig. 1). In vivo, overexpression of AM augments collateral flow in ischemic tissues partly through activation of endothelial nitric oxide synthase (eNOS) (1). Earlier studies have shown that the vasodilatory effects of AM are mediated by cAMP/protein kinase in smooth muscle cells (SMCs) (23) and by the eNOS/NO pathway in endothelial cells (17). Thus AM-induced angiogenesis and vasodilation may synergistically improve blood perfusion in ischemic tissues.

Recently, a seven-transmembrane G-protein-coupled receptor, calcitonin receptor-like receptor (CRLR), and receptor activity modifying proteins (RAMPs) have been recognized as integral components of the AM signaling system (38, 43). CRLR has demonstrated the expression of the transcript predominantly in microvascular endothelial cells. This finding supports the view that CRLR is potentially a major mediator of the effects of AM on the vasculature. The effect of AM on CRLR is modified by RAMP2 and RAMP3. The angiogenic effect of AM is mediated by CRLR/RAMP2 and CRLR/RAMP3 receptors (Fig. 1). VEGF and AM act synergistically to induce angiogenic-related effects on endothelial cells in vitro (11). However, blocking antibodies to VEGF cannot significantly inhibit AM-induced capillary tube formation by

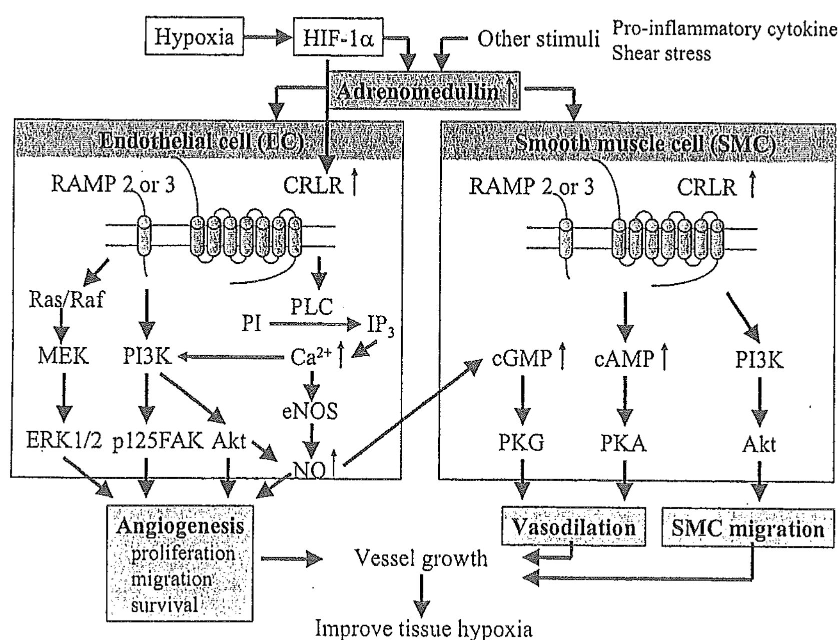

Fig. 1. Signaling pathway of adrenomedullin (AM) in vascular endothelial cells and smooth muscle cells. Both AM and calcitonin-receptor-like receptor (CRLR) are upregulated through a, hypoxia-inducible factor-1 (HIF-1)-dependent pathway under hypoxic conditions. AM binds to CRLR modified by receptor-activity-modifying protein 2 (RAMP2) and RAMP3. AM induces angiogenesis through activation of Akt, MAPK, and p125FAK in endothelial cells. AM also induces SMC migration and vasodilation. These activities synergistically improve tissue ischemia. MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; p125FAK, focal adhesion kinase; PLC, phospholipase C; PI, phosphatidylinositol; IP_3, inositol triphosphate; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; cGMP, guanosine 3′,5′-cyclic monophosphate; PKG, protein kinase G; PKA, protein kinase A.

HUVECs, indicating that AM does not function indirectly through upregulation of VEGF. Interestingly, AM and CRLR are both upregulated under hypoxic conditions in microvascular endothelial cells, although expression of RAMPs is not activated by hypoxia in microvascular cells (54). The activity of the CRLR promoter under hypoxic conditions is regulated at least in part through hypoxia-responsive regulatory element binding transcription factor HIF-1. Thus the simultaneous transcriptional upregulation of CRLR and its ligand AM in endothelial cells might play a significant role in the vascular responses to hypoxia and ischemia by creating a potent survival loop.

SMCs are essential for the generation of functional and mature blood vessels (26). We demonstrated in vivo that intramuscular administration of AM increased the number of αSMA-positive cells involved in the formation of vascular structures (25). In vitro, AM enhanced SMC migration, which was inhibited by wortmannin, a PI3K inhibitor. Recent studies using homozygous AM knockout mice have suggested that AM is essential for vascular morphogenesis (6, 21, 70). Taking these findings together, it is possible that AM contributes to vessel maturation through enhancement of SMC migration via a PI3K/Akt-dependent pathway (Fig. 1). This feature of AM-induced angiogenesis is different from VEGF-induced angiogenesis, which is not associated with vessel muturation.

In tumor cells, inflammation and hypoxia increase AM expression, and the elevated expression of AM is associated with tumor neovascularization in xenografted endometrial tumors and renal cell carcinoma (12, 86). AM also acts as a tumor cell survival factor underlying human carcinogenesis. Thus hypoxia-induced AM plays a part in tumor angiogenesis in conjunction with VEGF, and facilitates tumor growth under hypoxic conditions. As angiogenesis is an essential process in tumor-host interactions for tumor growth, maintenance, and metastasis, finding ways to regulate the action of AM may provide a new avenue for developing anticancer therapy (16).

THERAPEUTIC ANGIOGENESIS

A variety of studies have demonstrated that AM gene delivery serves as therapeutic tool to protect the cardiovascular system, including the heart (9, 32, 85), kidney (83), and vasculature (2, 84). In this section, we describe the angiogenic potential of AM gene transfer using novel gene delivery systems.

Nonviral gene transfer. Peripheral vascular disease is a crucial health issue affecting an estimated 27 million people (5). Despite recent advances in medical interventions, the symptoms of some patients with critical limb ischemia fail to be controlled. Although gene therapy has been shown to be an effective approach for angiogenesis (10, 24, 72), it is still unsatisfactory because of the biohazard of viral vectors, low transfection efficiency, and premature tissue-targeting. Therefore, highly efficient and safe gene transfer is desirable. Recently, we developed a novel nonviral vector, gelatin hydrogel, which allows highly efficient and long-lasting gene transfer (13, 30, 81). Gelatin has been widely used as a carrier of protein because of its capacity to delay protein degradation (76, 77). Plasmid DNA is known to be negatively charged. Thus we used gelatin as a vector for gene therapy. Biodegradable gelatin was prepared from pig skin. The gelatin was characterized by a spheroid shape with a diameter of ~30 μm, water content of 95% and an isoelectric point of 9 after swelling in water (76, 77). After 2-h incubation, positively charged gelatin held negatively charged plasmid DNA in its positively charged lattice structure. DNA particles are released from the gelatin through its degradation. As a result, DNA-gelatin complexes can delay gene degradation, leading to efficient gene transfer (13, 30, 44, 81).

We examined whether nonviral vector gelatin-mediated AM gene transfer induces therapeutic angiogenesis in a rabbit model of hindlimb ischemia (81). Seven days after intramuscular injection of AM DNA-gelatin complexes, there was intense AM immunoreactivity surrounding the gelatin in the skeletal muscles. AM production in the AM-gelatin group was enhanced compared with that in the naked AM DNA group, which received plasmid AM DNA alone. Unlike AM production in the naked AM group, AM overexpression in the AM-gelatin group lasted for longer than 2 wk. Importantly, AM DNA-gelatin complexes induced more potent angiogenic effects in a rabbit model of hindlimb ischemia than naked AM DNA, as evidenced by significant increases in histological capillary density, calf blood pressure ratio, and laser Doppler flow. These results suggest that the use of biodegradable gelatin as a nonviral vector augments AM expression and enhances AM-induced angiogenic effects. AM DNA-gelatin complexes were distributed mainly in connective tissues. It is interesting to speculate that the delay of gene degradation by gelatin may have been responsible for the highly efficient gene transfer. Thus gelatin-mediated AM gene transfer may be a new therapeutic strategy for the treatment of severe peripheral vascular disease.

Cell-based gene transfer. Recently, transplantation of stem cells or progenitor cells has been shown to regenerate a variety of tissues. Endothelial progenitor cells (EPCs) have been discovered in adult peripheral blood (4, 79). EPCs are mobilized from bone marrow into the peripheral blood in response to tissue ischemia or traumatic injury, migrate to sites of injured endothelium, and differentiate into mature endothelial cells in situ (15, 34). Transplantation of EPC induces therapeutic angiogenesis in the ischemic heart or limb (34, 42, 71). However, some patients are refractory to conventional cell therapy because of insufficient cell number, poor survival, or impaired differentiation. Thus a novel therapeutic strategy to enhance the angiogenic properties of EPCs is desirable. Considering the variety of protective effects of AM on vascular endothelial cells, we hypothesized that AM gene transfer into EPCs would strengthen the therapeutic potential of EPCs. Genetically modified EPCs may serve not only as a tissue-engineering tool to reconstruct the vasculature but also as a vehicle for gene delivery to injured endothelium.

Here, we present a new concept for cell-based gene delivery into the vasculature, consisting of three processes (44). First, positively charged gelatin is readily complexed with negatively charged plasmid DNA. Second, EPCs phagocytose ionically linked plasmid DNA-gelatin complexes in coculture, which allows nonviral gene transfer into EPCs with high efficiency. Third, intravenously administered gene-modified EPCs are incorporated into injured vascular beds. This novel gene delivery system has great advantages over conventional gene therapy; it is nonviral and noninvasive, and it provides highly efficient gene targeting into the vasculature. These benefits

may be achieved mainly by the capability of EPCs to phagocytose DNA-gelatin complexes and to migrate to sites of injured endothelium. Genetically modified EPCs markedly secreted AM into the culture medium, and AM overproduction lasted for more than 2 wk. The proliferative activity of AM DNA-transduced EPCs exceeded that of nontransduced EPCs. Furthermore, AM gene transfer inhibited apoptosis of EPCs in vivo and in vitro. Thus ex vivo AM gene transfer strengthened the therapeutic potential of EPCs.

Primary pulmonary hypertension (PPH) is a rare, but life-threatening disease characterized by progressive pulmonary hypertension, ultimately producing right ventricular failure and death (67). Median survival in patients with PPH is considered to be 2.8 years from the time of diagnosis. Thus novel and effective therapy is needed for the treatment of pulmonary hypertension. Because endothelial dysfunction may play a role in the pathogenesis of pulmonary hypertension such as PPH (3), pulmonary endothelial cells may be a therapeutic target for the treatment of pulmonary hypertension. We have demonstrated that administration of AM peptide decreases pulmonary vascular resistance in patients with PPH (45, 46, 48, 51). Thus we investigated the effects of AM gene-modified EPCs on pulmonary hypertension in rats (44). AM gene-transduced EPCs were similarly incorporated into the pulmonary vasculature. Immunohistochemical analyses demonstrated that the transplanted EPCs were of endothelial lineage and formed vascular structures. Intravenous administration of AM-expressing EPCs significantly decreased pulmonary vascular resistance compared with EPCs alone (−39%). Kaplan-Meier survival curves demonstrated that rats with pulmonary hypertension transplanted with AM-expressing EPCs had a significantly higher survival rate than those given culture medium or EPCs alone. These findings suggest that AM gene-modified EPCs using gelatin may serve not only as a tissue-engineering tool to reconstruct the pulmonary vasculature, but also as a vehicle for gene delivery to injured pulmonary endothelium. This hybrid cell-gene therapy may be applicable for intractable cardiovascular disease, including ischemic heart disease. Thus genetic manipulation of stem cells opens new avenues for regenerative medicine.

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シンクロトロンにかわる医用単色X線装置の開発と応用

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Development of monochromatic x-ray generators instead of a synchrotron and applications

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1. はじめに

X線レーザーの研究は世界各国で行われており，レーザー発振のためのさまざまな方法が考案されている[1-4]。レーザーは誘導放出による光の増幅を意味するが，誘導放出によってはフォトンエネルギーを高めることは難しい。このことから誘導放出とは異なるが，自由電子レーザー[5]による方法が考案され，研究されている。人体をも撮影できるハードX線レーザーの発振はノーベル賞に値するといわれているが，まだそれらの発生は報告されていない。したがって，もし仮にハードX線レーザーが定常的に発生できれば，医療における診断や治療に大きく貢献すると思われる。

シンクロトロンとモノクロコリメーターを用いて発生する単色平行X線はヨウ素のKエッジ造影

6-8) や位相コントラスト撮影 9-11) 等に応用され，X線撮影上革命的な成果をあげている。特に，血管造影にはK吸収端が 33.2 keV のヨウ素系造影剤が利用されるので，吸収端よりもわずかに高いフォトンエネルギーのX線は造影剤に効率良く吸収される。したがって，35 keV 程度の単色平行X線は微小血管造影には非常に有用であることから，造影室はシンクロトロン施設内に設置されている。

マイクロフォーカスX線管を用いた輪郭強調X線位相コントラスト撮影は Wilkins[12] により考案された。最近，100 μm 程度の小焦点モリブデン管と CR を用いた高精細マンモグラフィーシステム[13,14] がコニカミノルタから発売され，普及しつつある。X線撮像においてもデジタル化は進んでいるが，イメージングプレートを用いたコンピューターラジオグラフィー（CR）[15] やフラットパネルディテクター（FPD）はX線フィルムと比較して空間分解能が劣るので，拡大撮影による分解能の向上は必須である。さらに拡大により被写体からの散乱線の影響が低減され，位相コントラストの効果が加わる。このことから筆者等はデジタル拡大撮影の微小血管造影への応用を試み，良好な成果を得ている。

筆者等は単色X線撮影を行うため，エネルギー選択式の FPD の開発も行っているが，本稿では，近年開発した，種々のX線装置の特性や CR により撮影した画像について簡単に説明する。

2. 低フォトンエネルギープラズマX線装置

低フォトンエネルギーの弱電離プラズマX線装置[16-20] はハードX線レーザーの基礎研究のために開発され，銅やニッケル等のK系列特性X線を出力させるのに有用である。Fig. 1 のように 200 nF のコンデンサーを 50 kV 程度に充電し，蓄積された電荷をX線管の陰極にトリガ電圧を印加することにより放電する。この装置では高エネルギー放電により弱電離プラズマを成長させ，これを線状に形成することにより，制動X線が吸収され，蛍光X線（特性X線）に変換される。吸収係数が不連続なことから特性線はプラズマを容易に透過するので，単色化フィルターを挿入しなくとも高線量率の準単色X線が発生する。加えて，Kエッジフィルターにより Kβ線を吸収すれば，Kα線が得られる。X線管には長い棒状ターゲットが取付けてあり，1 mPa 程度に連続排気される。

管電圧と電流は減衰振動となり，それらの最大値は充電電圧を高めることにより増加した。実験結果より，管電圧の最大値は充電電圧にほぼ匹敵し，最大管電流値は約 15 kA であった。また熱蛍光線量計で測定した最大X線強度は線源から 1.0 m の位置で 1 パルス当たり 1.5 mGy 程度であった。

X線スペクトルの測定には，厚さ 0.5 mm のフッ化リチウム湾曲単結晶付の透過式分光器を用いた（Fig. 2)。実験ではクリーンなK系列特性X線が観測され，充電電圧の増加によりX線強度は著しく増加した。驚くことに，充電電圧が 50 kV では，高調波が観測された。

X線撮影には厚さ 10 μm のニッケルフィルターを用い，撮影距離と充電電圧はそれぞれ 1.2 m と 50 kV であった。写真はプラスチックの試験管からこぼれ落ちるプラスチック弾である（Fig. 3)。この装置のX線照射時間は約 1 μs であるため，完全静止画像が得られた。

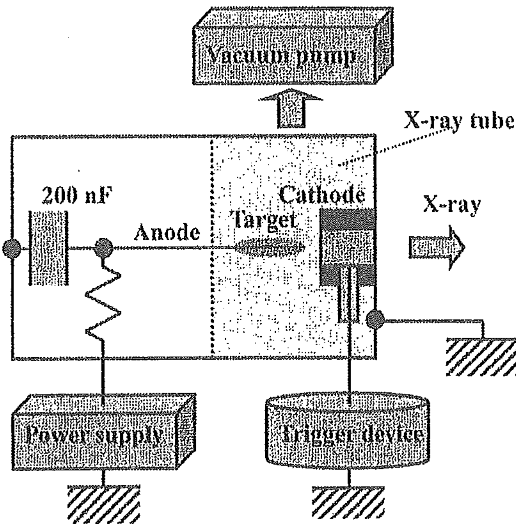

Fig. 1. Block diagram of the low photon energy plasma flash x-ray generator.

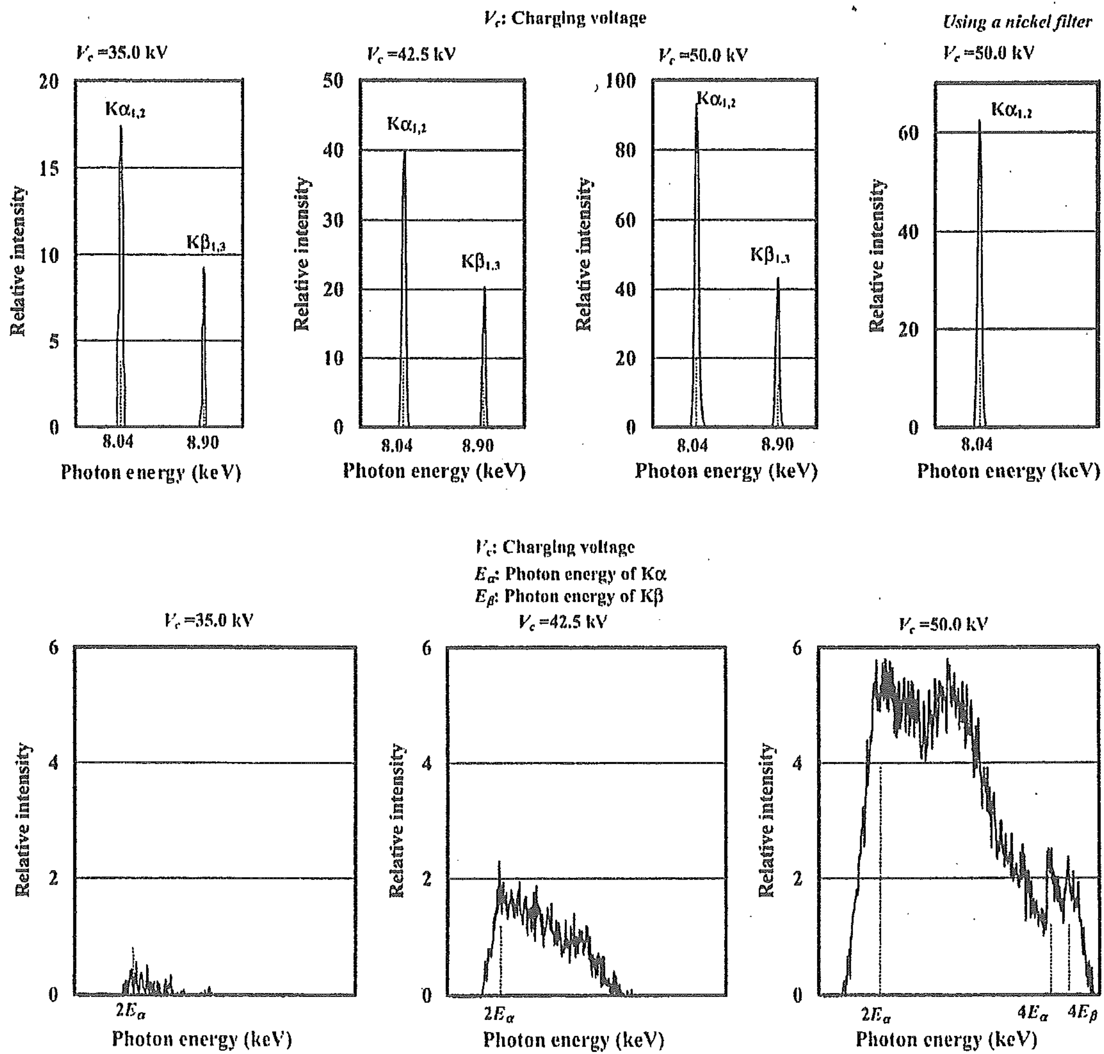

Fig. 2. X-ray spectra from weakly ionized linear plasma at the indicated conditions.

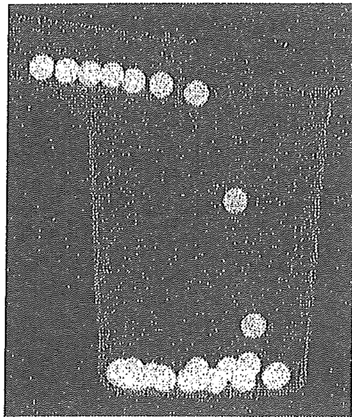

Fig. 3. Radiogram of plastic bullets falling into polypropylene beaker from a plastic test tube.

40 mm

3. 制動X線の角度分布を用いた単色フラッシュX線装置

この単色フラッシュX線装置[21,22]は高フォトンエネルギーのクリーンな特性X線を発生させるために試作した。制動線は電子軌道と反対方向には出力し難い性質を利用した場合には，比較的容易に準単色あるいは単色のX線を得ることができる。この装置は高電圧パルス発生装置，ターボ分子ポンプ，X線管などからなる。パルス発生装置では2段マルクス回路を採用し，充電電圧の約-2倍の高電圧パルスが出力する。X線管にはグラファイト製の円盤状陰極と棒状のモリブデンターゲットが付いており，陰極表面からの電子ビームがターゲット先端に衝突し，X線は陰極とマイラーX線窓を透過して出力する（Fig. 4)。管体はアクリル製で，1 mPa 程度に連続排気される。スペクトルは前述の結晶分光器を用いて測定した。厚さ 20 μm のジルコニウムフィルターを用いた場合にはKβ線が吸収されるので，クリーンな単色のKα線を得ることができた（Fig. 5)。フラッシュX線装置の管電圧と電流は減衰振動となり，それぞれの最大値は充電電圧が-70 kV の条件下で 120 kV と 1.0 kA であった。また熱蛍光線量計で測定した最大X線強度は線源から 0.5 m の位置で1パルス当たり 70 μGy であった。

Fig. 6はガラス製試験管から流れ出る水で，撮影距離と充電電圧はそれぞれ 0.5 m と-70 kV であった。X線パルスの幅は約 70 ns であるため，水の完全静止画像を撮影できた。

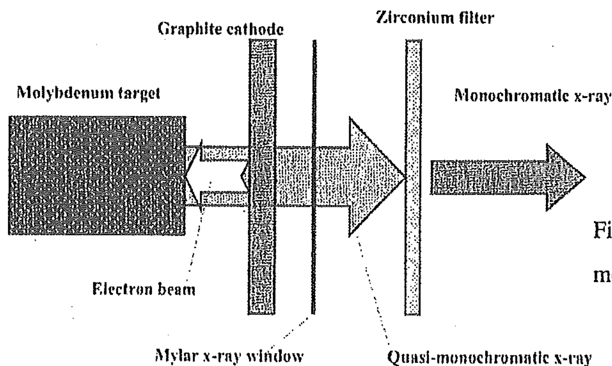

Fig. 4. K-photon irradiation using a monochromatic flash x-ray tube.

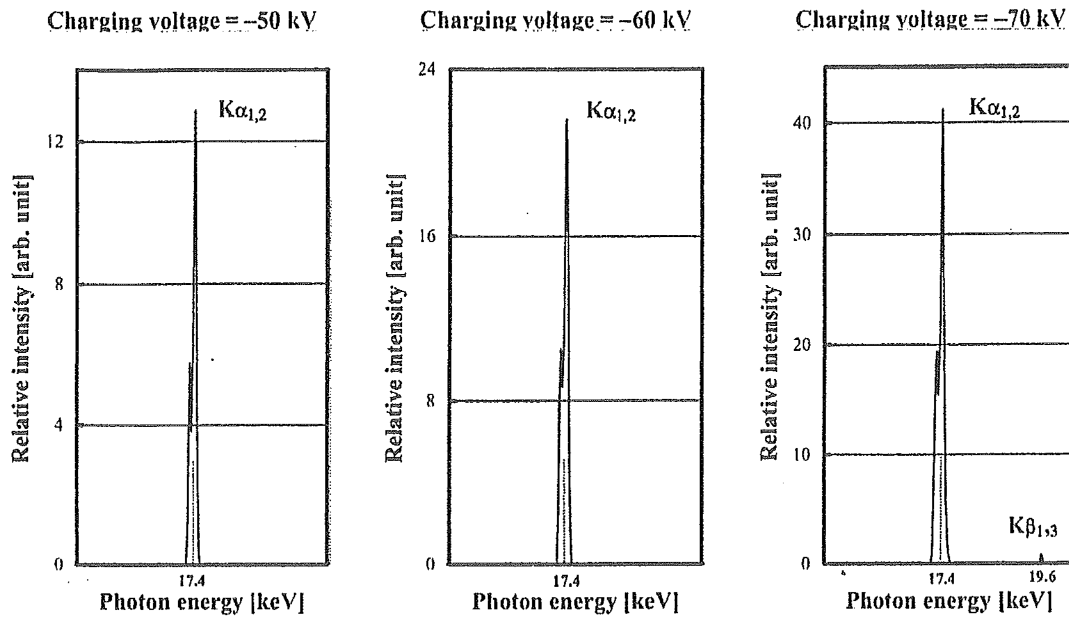

Fig. 5. X-ray spectra from a molybdenum target.

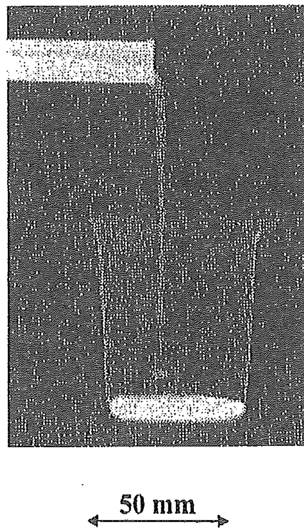

Fig. 6. Radiogram of water falling into polypropylene beaker from a glass test tube.

4. 高フォトンエネルギープラズマX線装置

高フォトンエネルギーのクリーンなK系列特性X線とそれらの高調波を発生させるため，高フォトンエネルギーのプラズマX線装置 23) を製作した。このフラッシュX線装置は高電圧充電器，コンデンサーユニット，ギャップスイッチ，ターボ分子ポンプ，そしてフラッシュX線管等よりなる。150 nFのコンデンサーを 80 kV 程度に充電し，蓄積された電荷をギャップスイッチを閉じることによりX線管内に放電し，フラッシュX線を得る。管はターボ分子ポンプにより 1 mPa 程度に連続排気される。

Fig. 7 は K 系列特性X線発生の原理図で，直径 3.0 mm の棒状タンタルターゲットと内径 4.5 mm のリング状グラファイト陰極が取り付けてある。陰極からの電子ビームはターゲット先端にほぼ垂直

に衝突するので，プラズマが形成され，図のようにK系列特性（準単色）X線が発生する。

ほぼ製品に近いプロトタイプゆえ，管電圧と電流の最大値を測定することは難しいが，充電電圧が 80 kV の場合の最大値はそれぞれ 160 kV と 40 kA 程度である。2極管を用いているので，X線照射時間は短く，約 100 ns 程度であった。次にフラッシュX線装置の線量率は極めて高いことから，半導体検出器を用いてスペクトルを測定することはできない。モリブデンターゲットから出力するX線スペクトルも結晶分光器を用いて測定したが，特性X線強度は充電電圧を高めることにより著しく増加した。しかし，20 μm 厚のジルコニウムフィルターを用いて Kβ線を吸収することは難しく，高調波も発生しなかった。一方，タンタルターゲットでも，ほぼクリーンなK系列特性X線を得ることができた (Fig. 8)。また熱蛍光線量計で測定した最大X線強度は線源から 1.0 m の位置で 1 パルス当たり約 300 μGy であった。

Fig. 9 はガドリニウムの質量吸収係数とタンタル Kα線の平均フォトンエネルギーの関係を示している。ガドリニウム造影剤は MRA で使用されるが，図のようにタンタル Kα線はガドリニウムに効率よく吸収される。実験結果から，重量百分率で 15 %程度の造影剤を用いれば，十分に高コントラストで撮影できる。酸化ガドリニウムを用いて造影したウサギ頭部を Fig. 10 に示す。図のように 100 μm 程度の微小血管が観察できた。

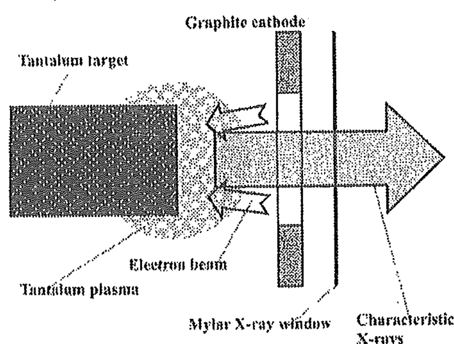

Fig. 7. K-photon irradiation from weakly ionized tantalum plasma.

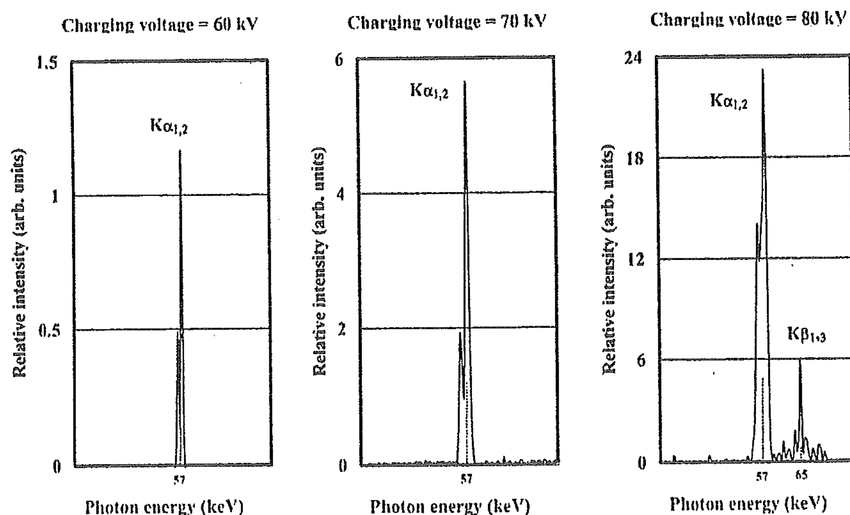

Fig. 8. X-ray spectra from a tantalum target.