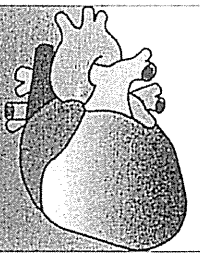
心臓手術の実際 第19回



外科医が語る術式，
麻酔科医が語る心臓麻酔，
臨床工学技士が語る体外循環法

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低侵襲心臓外科手術（MICS）における体外循環法

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ここでは，低侵襲心臓外科手術（MICS）における僧帽弁手術について解説する．当院では，1998年よりMICSを開始し，550例以上の症例に対し行ってきた．MICSにおいては確実な体外循環の確立，視野展開が何よりも重要であり，そのためには臨床工学技士，麻酔科医との連携が重要である．

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低侵襲心臓外科手術（MICS）における僧帽弁手術

1 低侵襲僧帽弁手術について

リウマチ熱の減少により僧帽弁狭窄症（mitral stenosis：MS）の発症頻度が低下している一方で，感染や変性疾患を主体とした僧帽弁閉鎖不全の症例は増加の一途をたどっている．

MSでは，左房からの血液の流出が妨げられるために房室間に圧差を生じ，血流の維持のために左房圧が上昇する．さらに上昇した左房圧が肺循環に伝わり，肺高血圧を呈する．そのため肺の間質に体液貯留を生じ，心不全症状を生じる．僧帽弁閉鎖不全では，左室の血液の一部が左房に駆出される．そのため，左房の拡大，左房圧の上昇，大動脈へ向かう拍出量の低下，拡張期に左房へ逆流した血流が左室へ戻ることによる左室の容量負荷を生じる．急性僧帽弁閉鎖不全では左房圧の上昇による肺水腫様の症状を呈し，慢性僧帽弁閉鎖不全では左房拡大が徐々に進行して左室の容量負荷が増大し，左心室の収縮能低下による低心拍出症状，心不全症状を呈する．

現在，僧帽弁閉鎖不全の症例においてはほとんどの症例で僧帽弁形成術（mitral valve plasty：MVP）が可能となりつつあり，僧帽弁閉鎖不全に対する治療戦略が，心不全既往のない症例や心房細動発症前・左心房拡大前というように，より早期での手術に移行する傾向にある．今後はMVPの治療成績はもとより，手術の低侵襲化という概念も重要である．1997年にChitwoodらは，従来のstandard MICS（minimally invasive cardiac surgery）よりさ

らに低侵襲化を目指したまったく胸骨の切開を行わないport-accessの手法を取り入れた，右第4肋間開胸での内視鏡下MVPを報告した[1]．当院でも1998年よりMICSを開始し，現在までに550例以上の症例に対し行ってきた．特に心房中隔欠損症，僧帽弁疾患に関してはMICSを第一選択としており，本稿では，MICSにおける僧帽弁手術について解説する．

2 当院における低侵襲僧帽弁手術の実際

2-1 皮膚切開から体外循環確立まで

体位は仰臥位，軽度右前斜位とし，麻酔は通常の気管挿管を行う．分離肺換気用のユニベントチューブを挿入している．右内頸静脈より「プリセップCVオキシメトリーカテーテル®」(エドワーズライフサイエンス(株))，脱血カニューレ挿入用に5 Frの「シースイントロデューサー」(東レ・メディカル(株))を挿入している．通常，皮膚切開は右乳腺下第4肋間で約5 cmの切開を行うが，女性患者では右乳房下縁を切開線とし，乳腺組織を上方に展開した後に第4肋間で開胸している[2]．片肺換気下に肋間開胸後，肺をよけ心臓の位置を心膜切開前に確認し，視野が狭すぎるようであれば上下どちらかの肋骨を肋軟骨部分で離断して視野を展開する．

体外循環は大腿動静脈からの経皮的心肺補助循環(percutaneous cardiopulmonary support：PCPS)用のカニューレ，および右内頸静脈からの脱血カニューレで確立する．送脱血カニューレ挿入はそれぞれ経食道心エコー(transesophageal echocardiography：TEE)モニタ下に確実に行っている．体外循環開始後，軽度脱血した部分体外循環下の状態で心膜を切開し，心膜および横隔膜を経皮的に牽引し皮膚に固定する(endoclose technique)ことで，視野およびworking spaceの展開を行う．脱血方法は陰圧吸引補助脱血法を採用しており，以前は1本の脱血カニューレのみで行っていたが，現在は右内頸静脈からの脱血カニューレを追加しており，上下大静脈のテーピングなしでも，左心房に肺静脈からの還流が多すぎて視野展開に難渋することはなく，良好な術野が得られる．

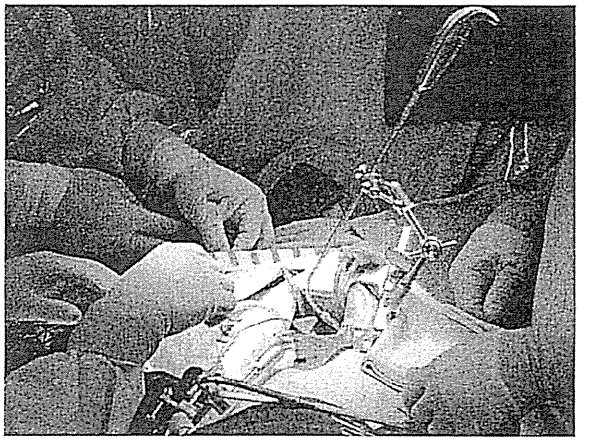

図1 「Adams-Yozu Mini-Valve System®」(UNIMEDIC Ltd.)

大動脈遮断鉗子としては，操作性の良さと簡便性を鑑み，「Cosgrove Flex Clamp®」(エドワーズライフサイエンス(株))を第一選択とし，どうしても視野の邪魔になりそうな場合などは「Modified Cosgrove Flex Clamp®」を術野以外の肋間から用いることとしている．

2-2 僧帽弁の展開

良好な視野展開は弁形成を成功させるための必要条件であり，特に左房・左室の無血視野が大切なことはいうまでもない．そこで，なるべく僧帽弁を正面視するために，僧帽弁への到達法は右側左房切開を原則にしている．左房切開の後に，自作のatrialリトラクター(鈎)を中隔にかけて上方に牽引し，僧帽弁を展開する．このリトラクターの把持には，以前はAESCULAP Inc.のエアサスペンション式のユニトラック・リトラクションシステムを用いていた．現在は我々がUNIMEDIC Ltd.と開発した「Adams-Yozu Mini-Valve System®」を用いている．このシステムの利点は，術野露出のためにさまざまな位置にリトラクターを把持することが可能であり，以前のものよりコンパクトな設計であるため術者，助手の心内操作を妨げずに術野を維持可能な点である[3](図1)．

右前胸部(第4肋間)の小切開は，深い術野

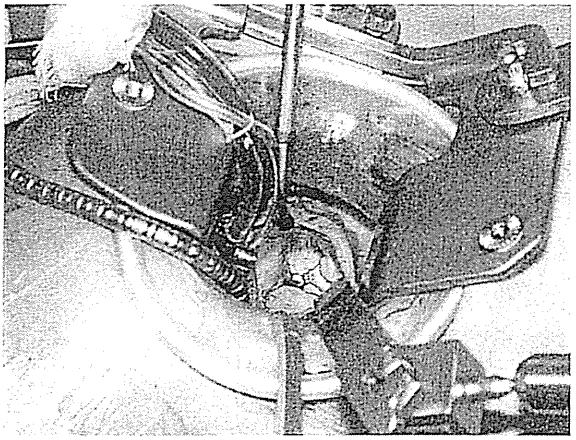

図2　術者からの視野

ではあるが，通常の胸骨正中切開より僧帽弁を正面視することが可能である．また，左房後壁・側壁の牽引（けんいん）には開胸器に固定して把持できるセルフリトラクターシステムを開発し，さらなる良好な視野と working space の確保が可能となっている（図2）．MICS における MVP では MICS 用の特別な機械を用いるが，僧帽弁形成法自体は通常の場合と同様である．

3 臨床工学技士が知っておくべき低侵襲僧帽弁手術に関連した周辺知識

3-1　確実な体外循環確立

体外循環は大腿動静脈，および右内頸静脈から挿入した PCPS 用のカニューレで確立するが，MICS の場合，安全・確実な体外循環の確立は非常に重要である．大腿動静脈よりのカニューレはカットダウンにて挿入しており，そのサイズは臨床工学技士と相談して決定している．大腿動脈の径が細い場合は，両側の大腿動脈から送血している．

送脱血カニューレ挿入時には，TEE にて先行させたガイドワイヤの位置が胸部下行大動脈内および右心房内にあることを確実に確認し，さらに大腿静脈から右心房内まで挿入する脱血カニューレは，ガイドワイヤを通して確実に右心房内に挿入されていることを TEE で確認している．MICS でこのような安全な体外循環確立を確実にするには，麻酔科医の協力が不可欠である．当院では，MICS に際しては，日本周術期経食道心エコー認定（Japanese Board of Perioperative Transesophageal Echocardiography：JB-POT）の資格をもつ麻酔科医が必ず立ち会うこととしている．そのため，手術中には麻酔科医から TEE を通しての多岐にわたる情報がリアルタイムにもたらされ，たいへん有用である．

3-2　大動脈遮断法・心停止・確実な心筋保護

大動脈遮断直後に大動脈基部に刺入したルートカニューレより心筋保護液を注入し心停止を行う．この場合の注意点は，何らかの理由で大動脈弁閉鎖不全の状態を発症し心筋保護液が左室に流れ込むことがあるため，必ず大動脈基部圧をモニタし，80 mmHg 以上に保つように確認すること，さらに心筋保護液注入中の大動脈基部を麻酔科医に TEE で描出してもらい，確実に大動脈弁が閉鎖し心筋保護液の注入が行われていることをモニタ画像で確認している．さらに，僧帽弁手術の場合は心筋保護液注入開始後，速やかに左房を切開し，減圧するよう心がけている．

3-3　体外循環の離脱

開胸心膜切開時より心嚢内に CO_2 2～3 L/min を吹き入れて心嚢内に残る空気を少なくするとともに，大動脈遮断解除後の空気抜きは，手術台をさまざまな角度に傾けることでルートカニューレや左房・左室に挿入したベントチューブから行い，麻酔科医により TEE にて残存空気の消失の確認をしてもらい，弁形成などの場合は形成の評価を施行してもらっている．

4 おわりに

当院においては，術前カンファレンスを外科医，心臓外科担当麻酔科医，臨床工学技士，手術室看護師同席の下で行っている．患者の病態については，チームで共通の認識をもって手術に臨むことが必要不可欠であると思われる．

MICSに対応した麻酔法

1 はじめに

MICSは1995年，米国オハイオ州クリーブランドクリニックで始められ，その定義は，①体外循環を用いない，②全胸骨切開を行わない，③その両方，のいずれかの条件を満たすものとされている．疾患別では，体外循環を用いるが全胸骨切開を行わない弁膜症手術や先天性心疾患の手術と，体外循環を用いない心拍動下冠動脈再建術などがある．本稿では，当院で行われている低侵襲法による僧帽弁手術に焦点を当て，その体外循環法と麻酔管理について述べる．

2 麻酔法

MICSの麻酔法は，従来の心臓麻酔と比べ，麻酔薬や循環作動薬の使用について特に大きな違いはなく，心機能や体外循環時間などの条件がそろえば早期抜管を試みる．MICSでは，胸骨非切開ないし小切開を行い，内視鏡支援下に手術を行うため，視野を良くする目的で片肺を虚脱させ，心膜切開や心臓の剥離を行う．このため，ダブルルーメンチューブ，もしくは気管支ブロッカーチューブ(ユニベントチューブなど)を用いて分離肺換気を行うことが要求される．当院では術後に気管内チューブに入れ替える際のリスクを避けるため，気管支ブロッカーチューブを用いている．

3 体外循環前のチェック

心臓血管手術を受ける患者では，動脈硬化病変を有することが多い．術前のCT画像や術中のTEE画像により，大動脈の動脈硬化や石灰化の程度，可動性のプラーク，動脈解離の評価を行い，大動脈のカニューレ挿入部位や遮断部位の変更を行う．上行大動脈の遮断部位は，TEEではblind zoneとなるため，上行大動脈近位部，大動脈弓部，下行大動脈に高度な動脈硬化病変が存在する場合は，上行大動脈に直接超音波探触子を当てる術野エコーで評価を行う．また，周術期にIABP(intra-aortic balloon pump)を留置することもあるが，動脈硬化病変が強い患者では禁忌となる．これらの病変を体外循環前に確認することは，脳塞栓症の予防に重要である[4]．また，左房内血栓，卵円孔開存，左上大静脈遺残など，術前に診断がついていなかった病変や合併奇形を見付けることで，体外循環の様式や術式を変更することもある[5]．

4 送血カニューレと脱血カニューレの挿入

MICSでは，小さな皮膚切開による手術を可能にするため，送血，脱血カニューレを末梢の大血管から挿入し，カテーテル類による術野の混雑を避ける．上行大動脈へのカニュレーションの代わりに大腿動脈に送血カニューレを挿入し，右房からの脱血は大腿静脈から経皮的に脱血カニューレを右房に挿入する．必要に応じて，右内頸静脈からの上大静脈カニューレと，大腿静脈からの下大静脈カニューレによる2本脱血とする．

送血，脱血カニューレの挿入は，TEEで下行大動脈や上・下大静脈を描出して，ガイドワイヤが正しく血管内に存在することを確認したうえで行う．大腿静脈からの脱血カニューレが肝静脈に迷入したり，心房中隔欠損孔や卵円孔開存を介して左房に迷入することがある．また，脱血カニューレがキアリネットワーク*1にぶつかって，下大静脈から右房に進まない場合もある．これらの所見はTEEで確認が可能である．左上大静脈遺残が認められる症例で，bridge veinがない場合や右房を切開する手術の場合は，冠静脈洞への3本目の脱血カニュー

*1 キアリネットワーク
下大静脈の右房への開口部から右心房壁や心房中隔を結ぶ線維状あるいは膜状の構造部で，右房内腫瘍と間違えられることがある．卵円孔開存や心房中隔瘤を合併することが多い．

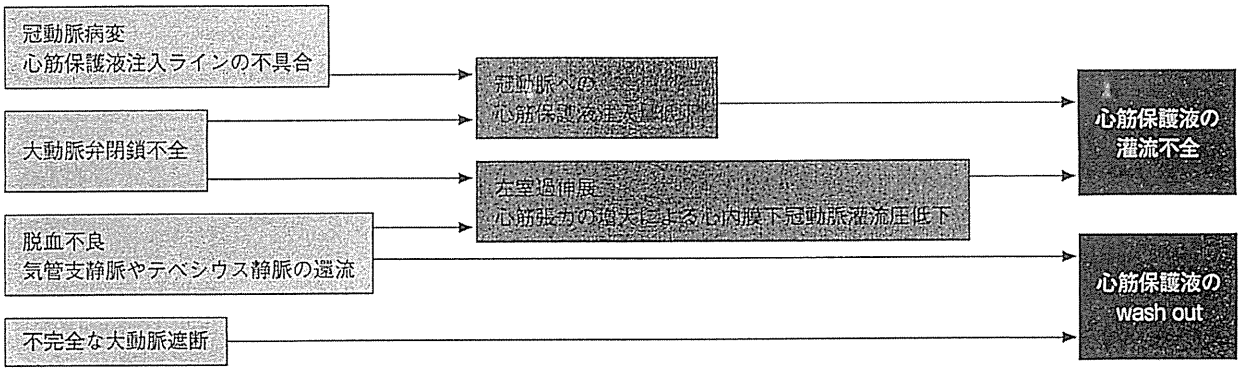

図3　心筋保護が不十分となる機序

レ挿入が必要となる．

5 心筋保護

心筋保護が不十分となる原因は，①心筋保護液の灌流不全と，②心筋保護液の wash out の2つに集約される．具体的には，①大動脈弁閉鎖不全，②冠動脈病変，③心筋保護液注入ラインの不具合，④脱血不良，⑤不完全な大動脈遮断，などがある．大動脈弁閉鎖不全は，冠動脈への心筋保護液の注入量の低下と左室の過伸展により心筋保護液の灌流不全を招く．脱血不良は，左室の過伸展に加え，心筋保護液を wash out してしまう（図3）．これらの異常の早期発見と速やかな対応が重要である．

心筋保護液注入時ならびに心内操作中の左室拡大を予防する目的で，心内ベントを左房，左室，肺動脈などに挿入するが，僧帽弁手術ではベントチューブは心腔内操作後に左室に挿入する．ベントチューブは心腔内遺残空気の除去や，体外循環離脱時に左室機能が十分に回復するまで左室仕事量の低減と左室の過伸展を予防する目的でも用いられる．逆行性冠灌流は，冠動脈高度狭窄病変や大動脈弁疾患で行われるが，左上大静脈遺残が認められる症例では，逆行性冠灌流は無効である．

6 心腔内遺残空気

自己心拍が再開し大動脈遮断が解除された後に，心腔内遺残空気を除去する．心腔内の遺残空気は，ほとんどすべての開心術で認められ，右上肺静脈，右冠動脈洞，左心耳，左室心尖部など心腔内の高いところに貯留しやすい．気泡型と貯留型があり，問題となるのは貯留型である．空気が右冠動脈に迷入して，心電図上 ST 変化や心室の壁運動異常が出現することがあるが，しばらく灌流圧を高めに保つことで回復する．手術台を傾けたり，心臓を揺すったりして，TEE により遺残空気の検索と誘導を行いながら，左房・左室ベントや大動脈基部ベントにより脱気する．開心中の術野での CO_2 使用も空気貯留を減らすうえで有用である．

7 体外循環からの離脱

体外循環からの離脱に際しては，残存逆流はどの程度か，溶血を引き起こす可能性はないか，弁狭窄が起こっていないかなど，手術結果の評価も並行して行う．復温や電解質のチェックを行い，心電図，動脈圧，肺動脈圧，中心静脈圧などの各種モニタで心機能の回復が確認できたら，徐々に心臓に容量負荷をかけ，自己心拍に移行していく．容量負荷により中心静脈圧が大きく上昇し，十分な動脈圧が得られない場合は，いったん脱血して人工心肺による補助循環を行い，心臓の負荷を軽減して，心機能の回復を待つ．体外循環からの離脱が困難な場合は，左室・右室の前負荷，収縮性，壁運動，各弁の機能などを，患者のベースラインと比較することが原因究明の有用な手がかりとなる．

8 まとめ

体外循環の管理を確実に行うことは，安全な手術を保証するうえで重要である．循環モニタ

の中でもTEEは大きな役割を担っており，術式の変更や手術結果の評価だけでなく，合併症の軽減や予後の改善も期待でき，MICSに必須のものとなっている[6),7)]．さらに本手術では，臨床工学技士とのコミュニケーションが必須であり，麻酔科医，心臓外科医，臨床工学技士の連携は手術成績を左右するだけでなく，患者の生命予後にも影響すると考える．

外科医 | 麻酔科医 | 臨床工学技士

MICSにおける体外循環法

1 当院における標準的体外循環法

当院での成人体外循環はすべて遠心ポンプ送血，陰圧吸引補助脱血(vaccum assisted venous drainage：VAVD)で行っている[8)]．また，静脈貯血槽バイパス回路があり，体外循環離脱時に静脈貯血槽の流入出口を閉じ，静脈貯血槽バイパス回路を開けることにより閉鎖回路となり，離脱操作を容易にしている．心筋保護装置には「MPS®」(QUEST Medical Inc.)を用いて，患者の心機能の状態に合わせた心筋保護を行っている(図4)．

2 MICSでの体外循環の実際

MICSでの体外循環と従来の心臓手術での体外循環に大きな違いはない．ただし，カニュレーションが末梢血管からとなる．

2-1 体外循環開始

確実なカニュレーションとACT(activated coagulation time)値が480秒を超えたことが確認できたら，体外循環を開始する．MICSでは，体外循環開始後，心膜切開が行われる．このとき適度の脱血により心房を虚脱した状態にする．VAVDでの陰圧の調節はゆっくりと行うことがコツである[9)]．適正灌流量の維持や脱血ができない場合は，脱血カニューレの位置を

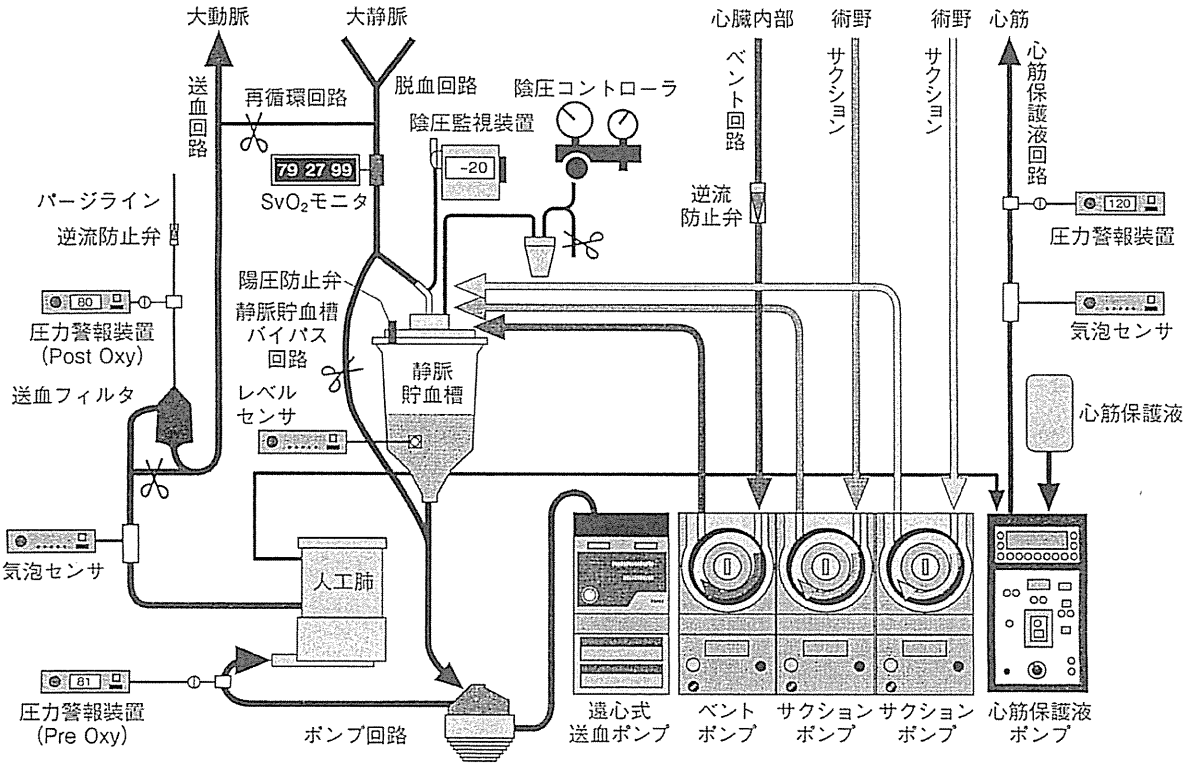

図4 慶應義塾大学病院における人工心肺システム

確認する.

2-2 大動脈遮断，心筋保護液注入

大動脈遮断時は人工心肺の血流量を低下させ，遮断確認後，ゆっくりと血流量を戻す．その後，心筋保護液を注入する．MICSでは切開創から大動脈が深いため，心筋保護液注入カニューレの長さを15 cmから20 cmにし，注入，ベント，圧測定が可能なカニューレを作製した．心筋保護液注入中は注入圧が80～90 mmHgになるように流量を調節する．注入圧が低い場合には，大動脈弁逆流が発生している可能性がある．術野に報告し，TEEで逆流がないか確認する[10].

2-3 心内操作中の体外循環

心房中隔欠損症例ではVAVDでの陰圧が弱すぎると下大静脈より血液が術野に流入し，無血視野は得られない．強すぎると無血視野は得られるが，脱血回路に大量の気泡が混入し，血液に悪影響を与える．適切な陰圧は，無血視野が得られ，脱血回路への気泡混入が微量な程度の陰圧に調節することである．そのためには術野ビデオモニタ，脱血回路の監視が重要である．一時的に脱血回路に気泡センサを装着し，気泡が検知しない程度に陰圧を調節することも1つの方法である.

僧帽弁症例では，視野を確保するために，左心房吊り上げ鈎(atrialリトラクター)により左心房を牽引する．このとき脱血不良になることがある[11]．その場合，術野に報告し，吊り上げ鈎の位置やカニューレの状態を確認してもらう．また，間欠的な心筋保護液注入時に注入圧が上昇しない場合がある．これは，左心房の牽引により大動脈弁が変形し，逆流が起きるからである[12]．心筋保護液注入時に注入圧が上昇しない場合には，左心房の牽引を一時的に解除し，心筋保護液注入後，再牽引する.

2-4 大動脈遮断解除

心内操作の終了が近付いたら復温を開始する．心内気泡除去のため，左房・左室ベント，大動脈基部ベントができるようにしておく．遮断解除時には血流量を低下させる．解除後，血流量をゆっくりと再開させながら，左房・左室ベント，大動脈基部ベントを開始する.

2-5 体外循環からの離脱

確実な気泡除去が確認されたら，まず左房・左室ベントを抜去する．その後，灌流圧(血圧)が50 mmHg程度になるまで脱血を行い，大動脈基部ベントを抜去する．抜去部分が確実に止血されていることを確認したら，ゆっくりと容量付加を行う．血行動態が安定したら徐々に血流量を下げていき，体外循環から離脱する.

3 MICSにおける体外循環のポイント

MICSにおける体外循環のポイントは，確実なカニュレーションと心筋保護と心内気泡除去である.

3-1 カニュレーション

MICSでの送脱血部位は，良好な視野を確保するために末梢の血管(大腿動脈，大腿静脈，内頸静脈など)が用いられる．そのため，カニューレはPCPSで用いられる経皮用カニューレが使用される．送血部位は大腿動脈である．カニュレーションはカットダウン(direct cutdown insertion)で行われる．ガイドワイヤ挿入時，確実に下行大動脈に挿入されているか，外科医，麻酔科医，臨床工学技士がTEEを観察して確認する.

カニューレは先端に側孔がある「FEM Ⅱ」(エドワーズライフサイエンス(株))を用いている．サイズは，体表面積1.5 m^2以下の症例では16 Fr，1.7 m^2以下では18 Fr，それ以上では20 Frを用いている．血流量が5 L/min以上の症例では，送血を2本に分け，両側の大腿動脈へ18 Frもしくは20 Frのカニューレを挿入して送血を行う．また，大腿動脈が細く16 Frが挿入できない症例では，両側の大腿動脈へ14 Frのカニューレ(小児用)を挿入して送血を行う．図5に両側送血用の回路を示す.

脱血部位は1本脱血では大腿静脈，2本脱血では内頸静脈と大腿静脈である．カニュレーションはdirect cutdown insertionかpercu-

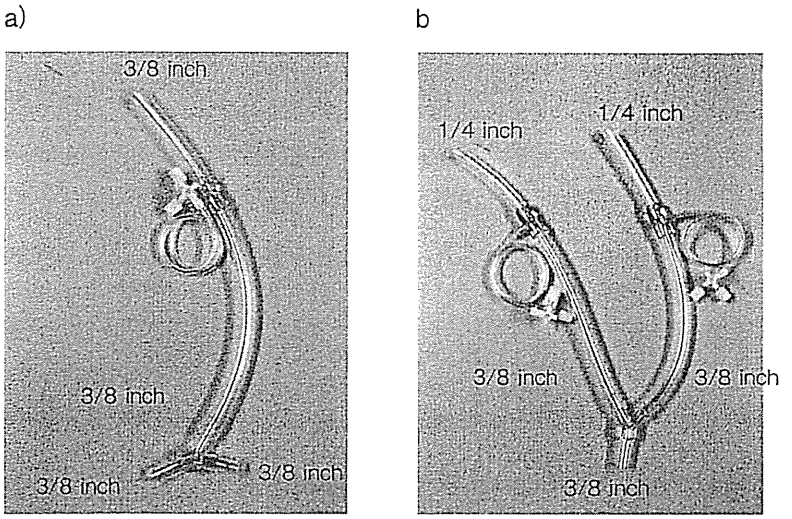

図5　両側送血用回路
a) 血流量 5 L/min 以上の場合.
b) 16 Fr のカニューレが挿入できない場合.

taneous insertion で行われる．どちらの場合もガイドワイヤ，ダイレータを用いてカニュレーションを行う．ガイドワイヤ挿入時，確実に右心房に挿入されているか，外科医，麻酔科医，臨床工学技士が TEE を観察して確認する．また，各サイズのダイレータを用いて確実に皮膚，血管孔を拡張させる．カニューレは側孔が多く，長さが 65 cm ある「VFEM」(エドワーズライフサイエンス(株)) を用いている．サイズは，体表面積 1.5 m^2 以下の症例では 18 Fr，1.6 m^2 以下では 20 Fr，1.7 m^2 以下では 22 Fr，それ以上では 24 Fr を用いているが，良好な脱血状態を維持するために，可能な限り太いカニューレを挿入する．また，脱血カニューレの位置も重要である．特に 1 本脱血の場合，カニューレの先端が上大静脈内まで挿入されなければならない．「VFEM」に附属しているダイレータは 16 Fr までしかないため，18 Fr 以上のカニューレを用いる場合は皮膚，血管孔の拡張が不十分である．そのため，18 Fr，20 Fr，22 Fr，24 Fr のダイレータを用意した．これにより，確実で安全なカニュレーションが可能となった．

3-2　心筋保護

MICS での心筋保護法は，当初，間欠的に順行性に投与する方法が主流であったが，現在では重症例の手術も行うため，順行性や逆行性など患者の状態に合わせた統合的な心筋保護を行っている．また，アイススラッシュなどを用いて局所冷却法を行っても，左心室の冷却は行えない．non-coronary collateral flow により大動脈遮断時でも心腔内に血液が満ち，常温体外循環の場合，心筋が温められてしまう．そのため，心筋保護の目的から体温を 28 ～ 30℃の中等度低体温体外循環を用いる．特に重要なのが注入時にカニューレの先端圧および TEE で逆流などがないか確認することであり，確実な心筋保護を行う．

3-3　心内気泡除去

MICS では，遮断解除後の心臓脱転や用手的な心臓圧迫による心内気泡除去は不可能である．そのため，心膜切開時から心嚢内に 2 ～ 3 L/min の CO_2 を吹送し，心内に残存する空気を少なくするとともに，手術台をいろいろな向きに傾け体位変換しながら，左房・左室ベント，大動脈基部ベントから十分時間をかけて気泡除去を行うことが重要である[13), 14)]．また，血流量を下げ，心臓に容量付加し，肺循環血液量を増加させることにより，肺静脈内の気泡が除去される．最終的に TEE で気泡がないことが確認できるまで，気泡除去を行う．

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Xenografted Human Amniotic Membrane–Derived Mesenchymal Stem Cells Are Immunologically Tolerated and Transdifferentiated Into Cardiomyocytes

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Rationale: Amniotic membrane is known to have the ability to transdifferentiate into multiple organs and is expected to stimulate a reduced immunologic reaction.

Objective: Determine whether human amniotic membrane–derived mesenchymal cells (hAMCs) can be an ideal allograftable stem cell source for cardiac regenerative medicine.

Methods and Results: We established hAMCs. After cardiomyogenic induction in vitro, hAMCs beat spontaneously, and the calculated cardiomyogenic transdifferentiation efficiency was 33%. Transplantation of hAMCs 2 weeks after myocardial infarction improved impaired left ventricular fractional shortening measured by echocardiogram (34±2% [n=8] to 39±2% [n=11]; $P<0.05$) and decreased myocardial fibrosis area (18±1% [n=9] to 13±1% [n=10]; $P<0.05$), significantly. Furthermore hAMCs transplanted into the infarcted myocardium of Wistar rats were transdifferentiated into cardiomyocytes in situ and survived for more than 4 weeks after the transplantation without using any immunosuppressant. Immunologic tolerance was caused by the hAMC-derived HLA-G expression, lack of MHC expression of hAMCs, and activation of FOXP3-positive regulatory T cells. Administration of IL-10 or progesterone, which is known to play an important role in feto-maternal tolerance during pregnancy, markedly increased HLA-G expression in hAMCs in vitro and, surprisingly, also increased cardiomyogenic transdifferentiation efficiency in vitro and in vivo.

Conclusions: Because hAMCs have a high ability to transdifferentiate into cardiomyocytes and to acquire immunologic tolerance in vivo, they can be a promising cellular source for allograftable stem cells for cardiac regenerative medicine. (*Circ Res*. 2010;106:1613-1623.)

Key Words: cardiomyogenesis ■ human mesenchymal stem cell ■ immunologic tolerance ■ myocardial infarction ■ cell-based therapy

Although embryonic stem cells[1] and induced pluripotent stem (iPS) cells[2] can be differentiated into cells of various organs, including cardiomyocytes, there are many underlining problems to overcome before clinical applications can be used, eg, tumorigenicity.[3] Autografts of iPS cells may not cause immunologic rejection; ironically, however, possible neoplasm formation would cause a serious problem because the neoplasm would not be rejected by the withdrawal of immunosuppressive agents. On the other hand, mesenchymal stem cells (MSCs) have recently been used for clinical application, and their safety and feasibility in cardiac stem cell-based therapy have been demonstrated.[4] Thus, MSCs are a more important cellular source for stem cell-based therapy from a practical point of view.

The efficacy of human bone marrow–derived MSCs (BMMSCs) was still limited,[5] however, because of low efficiency for cardiomyogenic transdifferentiation.[6] We previously reported that non–marrow-derived mesenchymal cells had higher cardiomyogenic transdifferentiation efficiency, eg, menstrual blood–derived mesenchymal cells (MMCs),[7] umbilical cord blood–derived mesenchymal stem cells (UCB-MSCs),[8] and placental chorionic plate–derived mesenchymal cells (PCPCs).[9] These cells are thought to be used by an allograft; therefore, problems of immunologic rejection arise. However, an allograft may be superior to an autograft in several ways. Taking into account the background condition of the patient (eg, metabolic disease or age),

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Non-standard Abbreviations and Acronyms	
AP	action potential
BMMSC	bone marrow–derived mesenchymal stem cell
EGFP	enhanced green fluorescent protein
FISH	fluorescent in situ hybridization
IL	interleukin
hAMC	human amniotic membrane–derived mesenchymal stem cell
hANP	human atrial natriuretic peptide
HLA	human leukocyte antigen
iPS	induced pluripotent stem
MHC	major histocompatibility complex
MI	myocardial infarction
MMC	menstrual blood–derived mesenchymal cell
MSC	mesenchymal stem cell
PCPC	placental chorionic plate–derived mesenchymal stem cell
PD	population doubling
UCB-MSC	umbilical cord blood–derived mesenchymal cell

stem cells obtained from young and healthy volunteers are expected to have a better efficacy in stem cell therapy.[10,11] Previously, mesenchymal cells did not express HLA-DR[6–9,12] and are believed to resist immunologic rejection to some extent. Shake et al[13] showed that xenografted mesenchymal cells were immunologically tolerated in the host heart. These cells, however, failed to show clear cardiomyogenic differentiation, and the mechanisms of tolerance were not well investigated. Unlike other mesenchymal cells (BMMSC, MMC, UCB-MSC, PCPC), only human amniotic membrane–derived mesenchymal stem cells (hAMCs) do not express the major histocompatibility complex (MHC) class I molecule and may be expected to show immunologic tolerance.

Recently, amniotic membrane–derived cells were reported to have potential for transdifferentiation into cells of various organs. Zhao et al,[14] and Miki et al,[15] reported evidence of possible cardiomyogenic transdifferentiation ability, but failed to show functioning cardiomyocytes. Fujimoto et al,[16] reported significant recovery of cardiac function by the rat amnion-derived cell transplantation in rat myocardial infarction (MI) model, however, they failed to show clear evidence of cardiomyogenic differentiation in vivo. Therefore, in the present study, we attempted to show: (1) the powerful cardiomyogenic transdifferentiation potential of our isolated hAMCs, and the beneficial effect of transplantation of hAMCs on cardiac function in vivo; (2) the induction of immunologic tolerance so that hAMCs can be a powerful allograftable stem cell source without either the administration of immunosuppressive agents or matching of MHC typing; (3) the mechanism of induction of tolerance; and (4) the close relationship between the cardiomyogenic transdifferentiation of mesenchymal cells and the process of immunologic tolerance.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Isolation and Culture of Human Amniotic Membrane–Derived Mesenchymal Cells

Human amniotic membrane was collected, with informed consent from individual patients, after delivery of a male neonate. The study was approved by the ethics committee of Keio University School of Medicine. The precise methods for culture have been described previously.[9,17] Detail is shown in the Online Data Supplement.

Coculture With Murine Fetal Cardiomyocytes

Cardiomyocytes were obtained from hearts of day-17 mouse fetuses.[6–9] The isolated cardiomyocytes were replated at $5\times10^4/cm^2$ on top of a floating atelocollagen film,[7,8] or on the bottom of a culture dish. The human amniotic membrane–derived mesenchymal cell (hAMCs) were infected with enhanced green fluorescent protein (EGFP)-expressing adenovirus.[6–9] The hAMCs were harvested and seeded on top of the cardiomyocytes (Figure 1H and 1I; Figure 2A, 2B, and 2H through 2M; Figure 3; Online Figure III) or on the bottom of the atelocollagen film (Figure 2D through 2F; Online Figure III) at $3\times10^3/cm^2$. For blocking experiments, neutralizing antibodies directed to HLA-G (clone 87G; Exbio) and IL10 (ab22771; Abacam) were added at a concentration of 20 μg/mL every day from 1 day before coculture till day 7. To prove the effect of immunologic reaction to the coculture system, we used the following immunosuppressive agents from one day before the coculture to day 7: FK506 (tacrolimus, 20 ng/mL, F4679, Sigma-Aldrich) and hydrocortisone (1 μg/mL, H0396, Sigma-Aldrich). In some experiment we used EGFP transgenic mouse fetuses (C57BL/6[18]).

Immunocytochemistry and Immunohistochemistry

A laser confocal microscope (FV1000, Olympus) was used for immunocytochemical analysis. Samples were stained with anti–cardiac troponin-I antibody or anti–sarcomeric α-actinin antibody and anti–connexin 43 antibody, as described previously.[7–9] In the present study, we used anti-FOXP3 antibody (IMGENEX, IMG-5276A), anti–HLA-G antibody (Abcam, ab7758), and anti-hANP antibody (YLEM, MCV928) according to the manufacturer's recommendation. The cells were isolated and stained immunocytochemically, and then observed by confocal laser microscope. The cardiomyogenic transdifferentiation efficiency was calculated as the fraction of cardiac troponin-I positive cells in the EGFP-positive cells.[7–9] Detail is shown in the Online Data Supplement. The methods to evaluate in vitro transdifferentiation potential to the noncardiac organs are provided in the Online Data Supplement.

Reverse Transcriptase–Polymerase Chain Reaction

RT-PCR was done as described previously.[6–9] PCR primers were prepared such that they would amplify the human but not the mouse genes. Primers for cardiomyocyte-related genes were used (see also Online Table I).

Western Blot and ELISA

Western blot analysis for hAMCs was done by iBlot dry blotting system (Invitrogen) according to the manufacturer's recommendation. Proteins were extracted from 1×10^6 hAMCs, placenta-derived mesenchymal cells, and menstrual blood–derived mesenchymal cells. Cellular lysates were electroblotted and probed using the anti–HLA-G IgG monoclonal antibody (1 μg/mL; EXBIO). Collected images were analyzed by the Image J software (http://rsbweb.nih.gov/ij/). The calculated data were normalized by the data of b-actin. Soluble HLA-G was measured by enzyme-linked immunosorbent assay (ELISA) in plates coated with the captured antibody MEM-G/09 (sHLA-G Kit; Exbio, Czech Republic), according to the manufacturer's recommendation. JEG3 (GeneTex, GTX14841) was used for the positive control of HLA-G.

Fluorescent In Situ Hybridization

The CEP X/Y DNA Probe Kit (Vysis) was used to determine the proportion of XX and XY cells according to the manufacturer's recommendation.[9] The Alu probe (BIOGENEX, PR-100101) was used according to the manufacturer's recommendation.

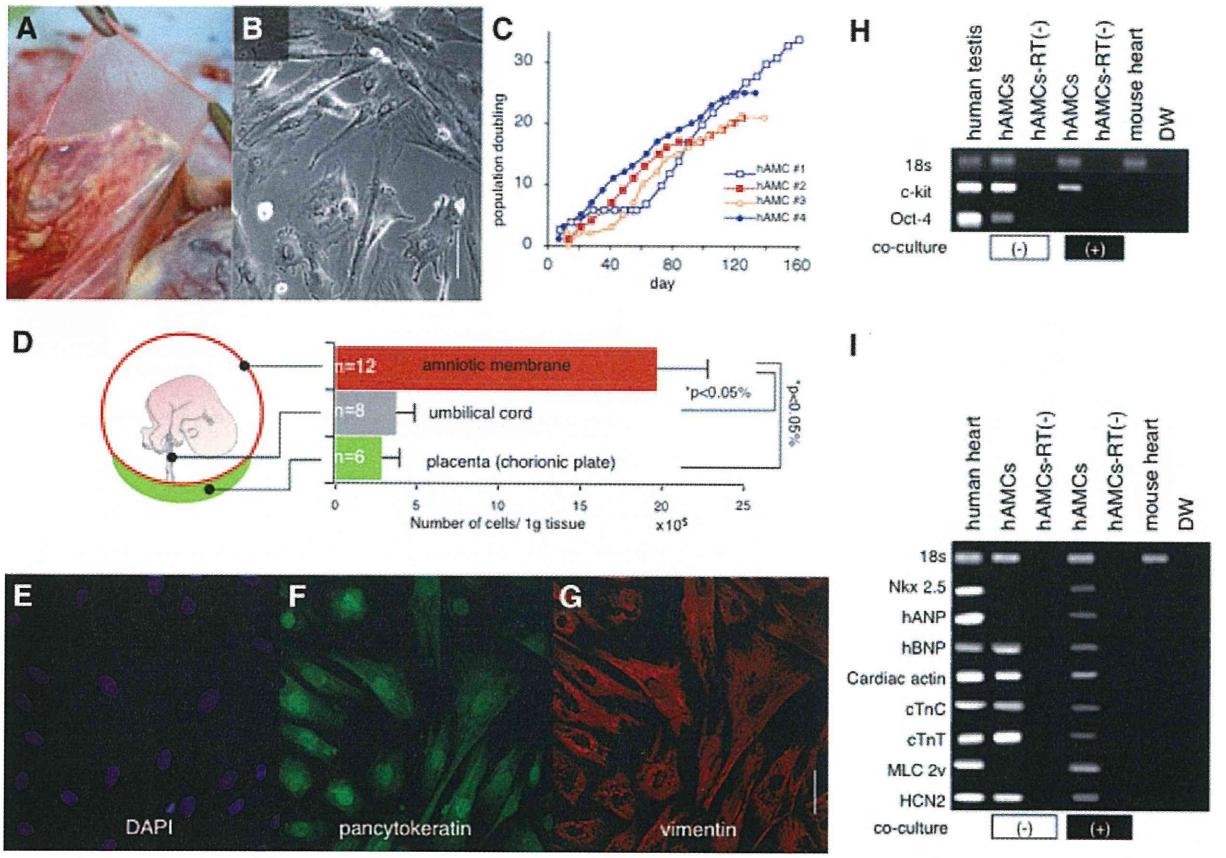

Figure 1. Cellular phenotype, surface marker, and expression of cardiomyocyte-specific genes. A, Macroscopic view of amniotic membrane. **B,** Phase contrast microscopic view of hAMCs. **C,** The representative growth curves of hAMCs as a function of time after the culture. **D,** Numbers of collected mesenchymal cells obtained from 1 g of placental tissues at 2 weeks after the start of culture are shown. n indicates number of delivery. **E through G,** Laser confocal microscopic view of immunocytochemistry of hAMCs in culture with anti-pancytokeratin **(green; F)** and anti-vimentin **(red; G)** antibodies. **H and I,** RT-PCR was performed with PCR primers with specificity for human genes encoding cardiac proteins but not for the corresponding murine genes (Online Table I) before (−, **white box**) and after (+, **black box**) the coculture. **H,** RT-PCR for stem cell markers was performed. hAMCs express Oct-4 and c-kit at the default state. **I,** Human heart cells and mouse heart cells were used as a positive control and negative control, respectively. Most human cardiac genes were expressed after the coculture. **Scale bars:** 20 μm **(B and G).**

Physiological Analysis

Functional analysis was performed at 1 week after the cardiomyogenic induction. The method of action potential (AP) recording was as previously described.[6–9] Alexa 568 was injected into cells via recording microelectrodes to stain the cells and confirm that the AP was generated by EGFP-positive cells (Figure 1B). Contraction of the cells was measured by the video image of EGFP-positive cells as described previously.[7–9]

hAMC Transplantation in Myocardial Infarction Model In Vivo

Myocardial infarction (MI) was induced in the open chests of anesthetized Wistar rats (8 weeks of age) or of F344 nude rats (Clea Japan Inc) (6 weeks of age), as described previously.[7,19] Two weeks after the MI, 1 to 2×10^6 of EGFP-labeled hAMCs were injected into the myocardium at the border zone of the MI[7]; they survived without using an immunosuppressive agent. In some experiment EGFP-transgenic mouse[18] was uses as recipients. To examine the effect of hAMC transplantation in vivo, we selected nude rats as recipients.[7] Two weeks after the first operation, nude rats with MI were randomized in a blind study of the following groups: the control MI group (MI), the MI+hAMC transplanted group (MI+hAMC), and the sham operated group (sham). Randomization occurred immediately before echocardiogram. Immediately before cell transplantation, 2D and M-mode echocardiographic (8.5-MHz linear transducer; EnVisor C, Phillips Medical System, Andover, Mass) images were obtained to assess left ventricular end-diastolic dimension and left ventricular end-systolic dimension at the midpapillary muscle level. Two weeks after the transplantation, a similar echocardiogram was performed again. ECG and left ventricular pressure were measured as previously described.[7] Tissue samples were obtained by slicing along the short axis of the left ventricle, for every 1 mm of depth. After Masson's trichrome staining, the area of fibrosis was digitized from each slice, and then the percentage of fibrosis area in the left ventricular myocardium was calculated, as previously described.[7] To test the potential induction of immunologic tolerance, hAMCs were transplanted into the Wistar rats. Hearts and sera were obtained at between 2 days to 56 days after hAMC transplantation, and then analyzed by immunohistochemistry, fluorescent in situ hybridization (FISH), and ELISA experimental methodologies. The survival rate of EGFP-positive cardiomyocytes was calculated by fluorescent microscope (details are shown in the Online Data Supplement). In some experiments, hAMCs were pretreated with 10 ng/mL of IL10 (Sigma I9276) or 10 ng/mL of progesterone (p7556; Sigma-Aldrich) for 2-days before the transplantation to observe the efficacy of survival of hAMCs in vivo. Enzymatically isolated EGFP-positive cardiomyocytes[20,21] were selected by glass pipette driven by a manipulator mounted on the inverted fluorescent microscope, then used for the FISH experiment to determine the origin of EGFP-positive transdifferentiated cardiomyocytes. See also the Online Data Supplement.

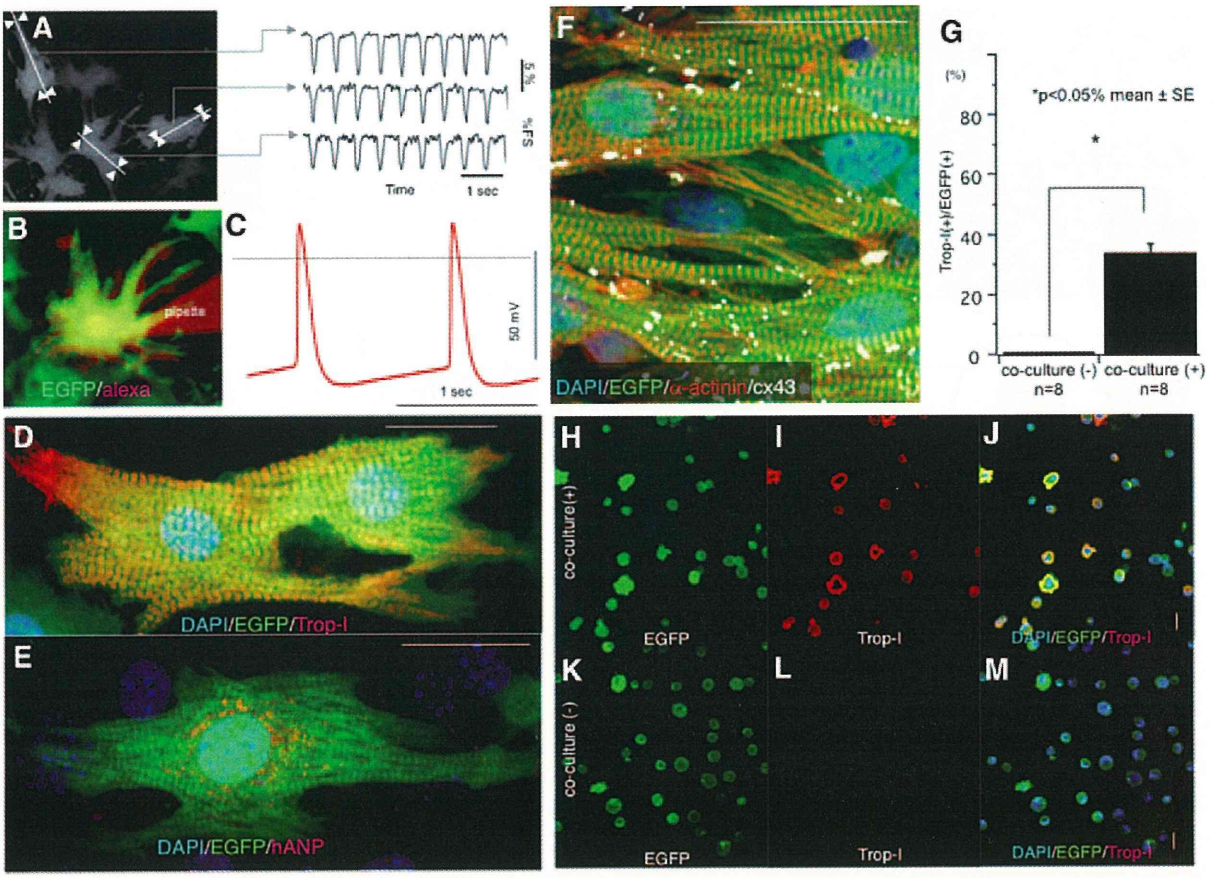

Figure 2. Cardiomyogenic differentiation of hAMCs in vitro. A, Still image **(left)** and detected fractional shortening (%FS), along the **white lines** between the **white arrows**, are shown in the **right panel. B,** EGFP-labeled hAMCs were injected with Alexa 568 solution **(red)** through microelectrodes to confirm that the recorded signal was obtained from hAMCs. **C,** Representative AP traces are shown (horizontal line denotes 1 second). The vertical line denotes 50 mV and the dotted horizontal line denotes 0 mV. **D through F,** Laser confocal microscopic view of immunocytochemistry of differentiated hAMCs with anti–cardiac troponin I (Trop-I) **(D)**, anti-human atrial natriuretic peptide (hANP) **(E)**, anti–sarcomeric α-actinin (α-actinin) **(F)** (see also Online Figure III), and anti–connexin 43 (Cx43) **(F)** antibodies. EGFP-positive **(green)** hAMCs expressed troponin I **(red)**, hANP **(red)**, α-actinin **(red)**, and Cx-43 **(white)**. Nuclei were stained with DAPI **(blue)**. **Scale bar:** 20 μm. Clear striation pattern of troponin I and α-actinin, and diffuse dot-like staining of connexin 43 and hANP were observed. **G,** Cardiomyogenic transdifferentiation efficiency before (−) and after (+) the coculture of hAMCs. **H through M,** Representative immunocytochemical images of troponin I for enzymatically isolated differentiated hAMCs with and without coculture. **Scale bar:** 20 μm.

Statistical Analysis

All data are shown as the mean value ± SE. The difference between two mean values was determined with a Student *t* test. The difference among more than 3 mean values was determined with one-way ANOVA test or one-way repeated measures ANOVA test and Bonferroni post hoc test. Statistical significance was set at $P<0.05$.

Results

This study was approved by the institutional ethical committee. With informed consent, human placenta and amniotic membrane were collected after delivery of a male neonate. The amniotic membrane was pealed off from the maternal placenta (Figure 1A) and hAMCs (Figure 1B) were collected by the culture method, as described previously.[9,17] The hAMCs proliferated at 18 to 22 population doublings (PDs) (Figure 1C), and experiments were performed on hAMCs at 2 to 9 PDs unless otherwise mentioned. The number of mesenchymal cells at 3 days after the primary culture per 1g tissue samples from the amniotic membrane, umbilical cord, and placenta[9] are shown in Figure 1D. This suggests that the amniotic membrane is a rich cellular source of mesenchymal cells. FISH analysis for human chromosome X and Y revealed that 100% of the obtained cells were of male infant origin. Immunocytochemical analysis revealed that hAMCs expressed both pancytokeratin and vimentin, suggesting hAMCs have both epithelial and mesenchymal phenotypes (Figure 1E through EG). Surface marker analysis (Online Figure I) revealed that hAMCs did not express hematopoietic linage markers, eg, CD14, CD34, CD45, CD117, and CD309, and did express mesenchymal linage markers, eg, CD10, CD29, CD44, and CD105. It is noteworthy that hAMCs were positive for SSEA4,[22] an embryonic stem cell marker. hAMCs were negative for HLA-ABC, HLA-D, and HLA-DR. The RT-PCR was performed with primers that hybridized with human-specific genes but not with the murine orthologs.[6–8] The hAMCs expressed Oct-4 and c-kit (Figure 1H) and did not express Nkx2.5 before cardiomyogenic induction (Figure 1I). Almost all cardiac-specific genes were expressed after cardiomyogenic induction (Figure 1I).

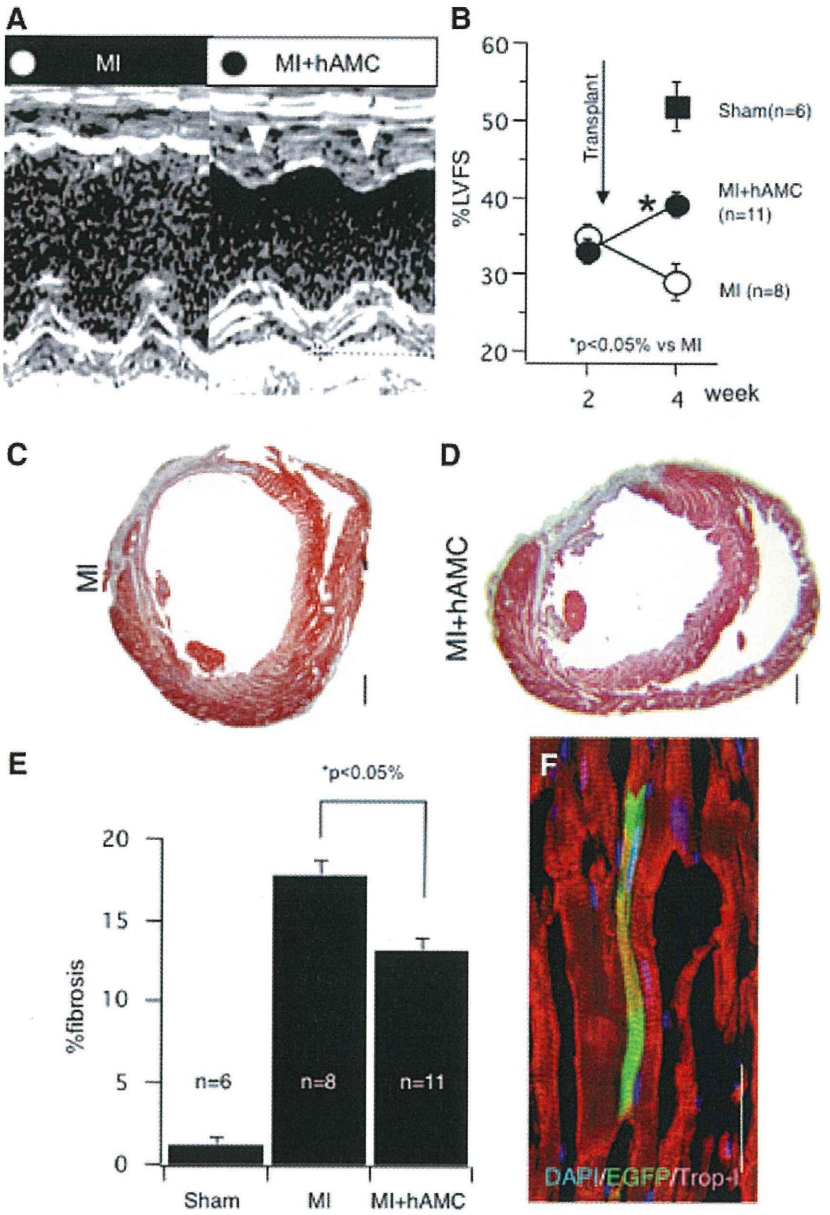

Figure 3. Effect of hAMC transplantation on heart function in vivo. A, Representative traces of M-mode echocardiogram from the control MI of nude rats and MI+hAMC transplanted nude rats (MI+hAMC) are shown. The contraction of the left ventricular anterior wall was improved by transplantation of hAMCs **(white arrows). B,** The hAMC transplantation significantly increased in measured percentage of left ventricular fractional shortening (%LVEF) at 4 weeks after the first operation. **C and D,** Representative Masson's trichrome staining of the heart at the papillary muscle level is shown. The digitized data were measured and calculated in **E**. The hAMC transplantation significantly decreased in percentage of fibrosis area. **Scale bar:** 1 mm. **F,** Merged image of confocal laser microscopic view of immunohistochemistry with anti–cardiac troponin I antibody (Trop-I) **(red)**, DAPI **(blue)**, and EGFP **(green)**. The EGFP-positive hAMC-derived cardiomyocytes were observed at the margin of the MI area of the nude rats. **Scale bar:** 50 μm.

Cardiomyogenic induction of hAMCs was performed by a coculture system with fetal murine cardiomyocytes, as described previously.[6–9] EGFP-labeled hAMCs started beating within a few days after the induction and about half of the hAMCs spontaneously contracted in a synchronized manner (Figure 2A); the averaged percentage of fractional shortening (%FS) was 6.2±0.6% (n=8). The recorded AP from EGFP-positive hAMCs (Figure 2B) showed pacemaker-like potential (n=7) and cardiomyocyte-specific long AP duration (Figure 2C). Averaged amplitude was 71.5±2.2 mV, maximal diastolic potential was -52.7±1.9 mV, AP duration at 90% repolarization was 161.6±9.3 ms, and beating cycle length was 1.06±0.1 s (n=7). Cardiomyogenic transdifferentiation could be observed when the murine cardiomyocytes and hAMCs were separately cocultured by the atelocollagen membrane (Figure 2D through 2F; Online Figure III) that is permeable for only small molecules (less than 5,000MW).[7,8] In another experiment murine cardiomyocytes were stained with MitoTracker Red (Invitrogen, M7512) and cocultured with hAMCs, then we confirmed that almost all EGFP positive cardiomyocytes were MitoTracker negative (Online Figure II, A through E). On the other hand hAMCs were stained with the MitoTracker Red and cocultured with EGFP-transgenic murine cardiomyocytes, then we confirmed that almost all MitoTracker positive cardiomyocytes were EGFP negative (Online Figure II, F through J). Thus, we concluded that the observed EGFP-positive cardiomyocytes were caused not by cell fusion between murine cardiomyocytes and hAMCs, but by transdifferentiation of hAMCs. Immunocytochemical analysis revealed a clear striation pattern of cardiac troponin-I (Figure 2D), dot-like pattern of human atrial natriuretic peptide (hANP) (Figure 2E), clear striation

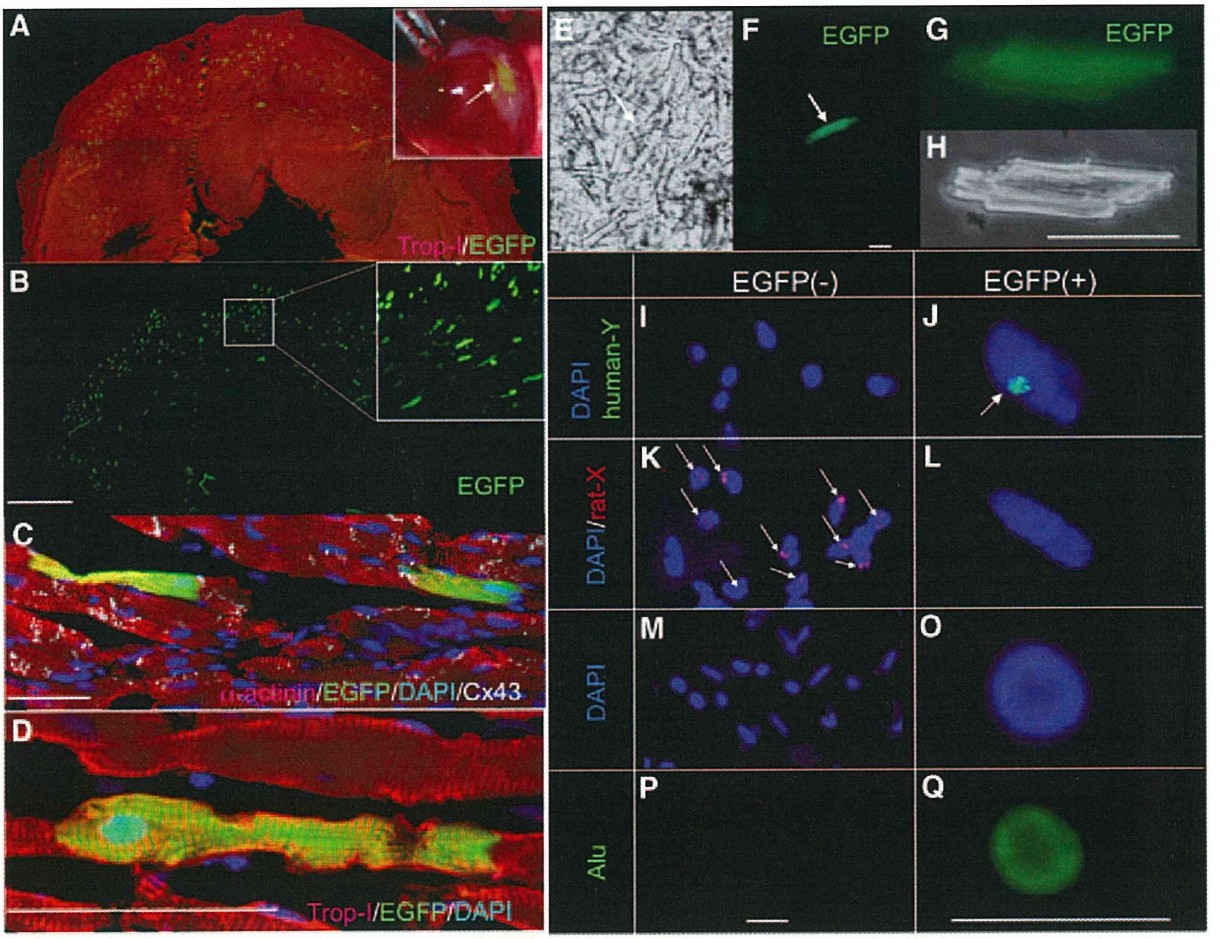

Figure 4. Survival of xenografted hAMCs transdifferentiated into cardiomyocytes in vivo. EGFP-labeled hAMCs were transplanted into the myocardium at the margin of MI of Wistar rat hearts (**white arrow** in the inset of **A**) and survived for 2 weeks. **A and B,** Fluorescent microscopic view of immunohistochemistry of left ventricle at 2 weeks after the transplantation with anti–cardiac troponin-I (Trop-I) **(red) (B)** EGFP **(green)** and **(A)** merged image with EGFP and troponin I. Significant numbers of EGFP-positive cells were observed at the margin of MI by lower magnification view **(B)** (see also Online Figure VI; Figure VII, F through J). **C and D,** Laser confocal microscopic view of immunohistochemistry of differentiated EGFP-positive hAMCs (4 weeks after transplantation) with anti–sarcomeric α-actinin (α-actinin) **(red; C)**, anti–connexin 43 (Cx43) **(white; C)**, and anti–troponin I **(red; D)** antibodies (see also Online Figure VII, A through E). **E through H,** Phase contrast image **(E and H)** and fluorescent image **(F and G)** in the same visual field of cardiomyocytes immediately after enzymatic isolation **(E and F)** and after selection of EGFP-positive cardiomyocytes **(G and H)**. **I through Q,** FISH analysis for human Y chromosome **(green; I and J)**, rat X chromosome **(red; K and L)**, and human-specific Alu **(green; P and Q)** of EGFP-negative **(I, K, M, and P)** and EGFP-positive **(J, L, O, and Q)** cardiomyocytes. Nuclei staining by DAPI **(blue)** in the same visual filed of **P and Q** are shown in **M and O**, respectively. EGFP-positive cardiomyocytes were of human origin. **Scale bars:** 1 mm **(B)**; and 50 μm **(C, D, F, and H)**; 20 μm **(P and Q)**.

pattern of α-actinin, and dotted staining of connexin 43 (Figure 2F and Online Figure III). The percentage of cardiac troponin-I positive cells in the EGFP-positive cells was defined by immunocytochemical analysis (Figure 2G through 2M) and calculated to determine the cardiomyogenic transdifferentiation efficiency.[7–9] The efficiency was significantly increased up to 33±3% (n=8) by the cocultivation.

The hAMCs were transplanted into the hearts of nude rats with chronic MI, in vivo, and the effect on cardiac function was examined. Echocardiography showed a significant increase in the left ventricular fractional shortening (%LVFS) at 2 weeks after transplantation (Figure 3A and 3B; see also Online Figure IV). The heart section was stained with Masson's trichrome (Figure 3C and 3D) and the MI area was digitized and measured (Figure 3E). The percentage of fibrosis area was significantly decreased by hAMC transplantation (MI n=8, MI+hAMC n=11, $P<0.05$). The EGFP-positive cells of hAMCs (Figure 3F) observed at the MI area expressed a clear striation staining pattern of cardiac troponin-I, suggesting in situ cardiomyogenic transdifferentiation ability for hAMCs. The rate of survived EGFP-positive cardiomyocytes was 1.125±0.470% (n=6).

We tested whether xenografted hAMCs may be immunologically tolerated to survive for more than 2 weeks and differentiate into cardiomyocytes in situ. Isolated hAMCs were injected into the MI zone of female Wistar rats (Figure 4A, inset, white arrow). A significant number of EGFP-labeled rod-shaped cardiomyocytes were observed (Figure 4A and 4B), even 2 weeks after the transplantation (at least 80 days; Online Figure V). Immunohistochemistry revealed that

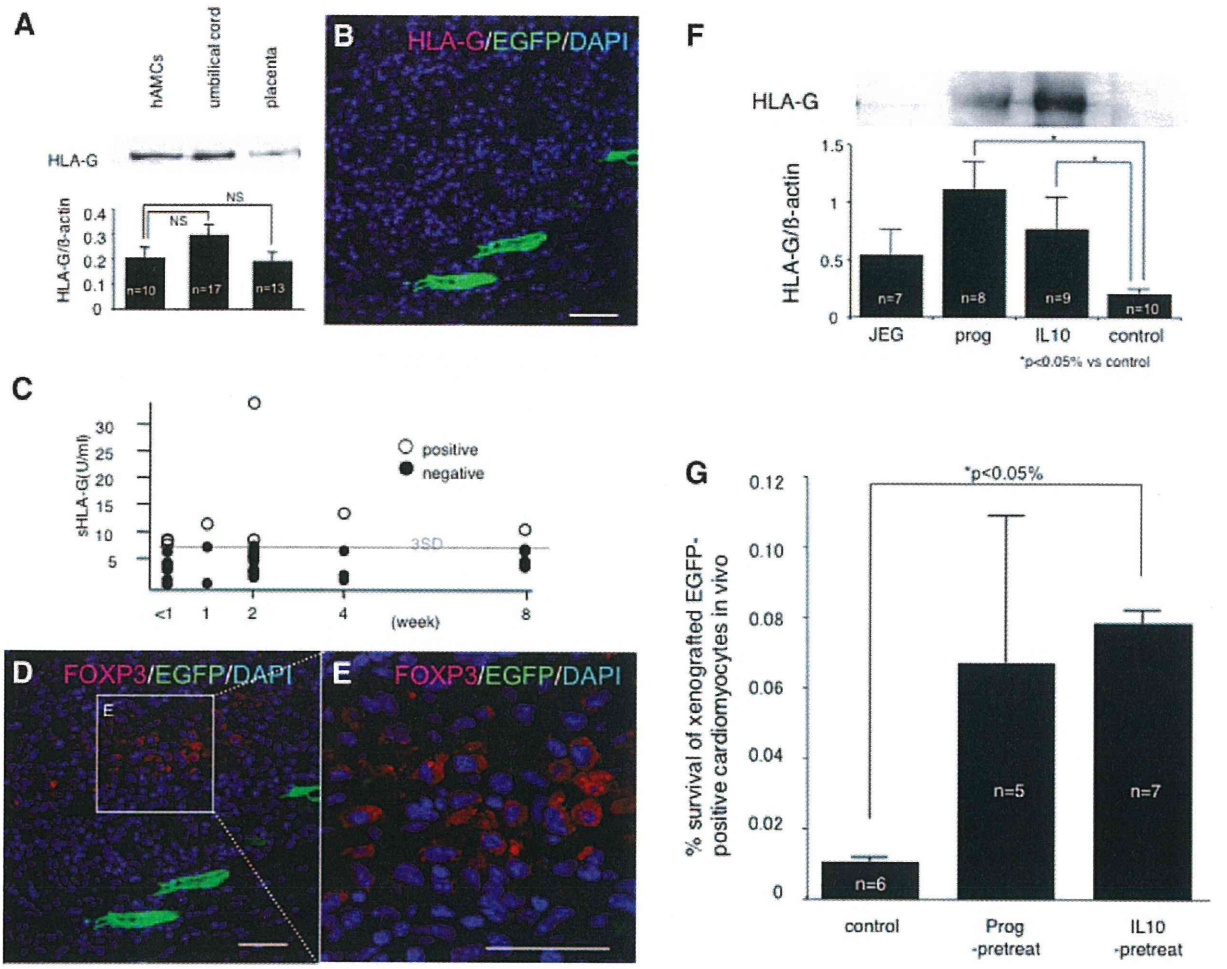

Figure 5. Role of HLA-G and regulatory T cells in immunologic tolerance of xenografted hAMCs. A, Western blot analysis of HLA-G in the mesenchymal cells obtained from amniotic membrane (hAMCs), umbilical cord, and placenta. Densitometry analysis of HLA-G, normalized by β-actin. **B,** Laser confocal microscopic view of immunohistochemistry of host myocardium (2 weeks after transplantation) with anti–HLA-G antibody **(red)**. The transdifferentiated EGFP-positive cardiomyocytes did not show any membrane biding isoform of HLA-G in situ. **C,** Concentration of the soluble form of HLA-G (sHLA-G) in the sera of hAMC transplanted Wistar rats detected by ELISA as a function of time in weeks after the transplantation is shown. There is no correlation between the survival of EGFP-positive cardiomyocytes and sHLA-G concentration. **D,** Immunohistochemistry with anti-FOXP3 **(red)** antibody. **E,** Expansion of **area within the white box** in **D** (see also Online Figure IX). A significant number of FOXP3-positive regulatory T cells have migrated beside the hAMC-derived cardiomyocytes. **F,** The effect of agents related to fetomaternal immunologic interaction on the HLA-G expression in the hAMCs was tested by Western blot analysis. Densitometric data were normalized by b-actin. Both IL10 and progesterone (prog) markedly increased the HLA-G expression in hAMCs. Concordant with **F**, pretreatment with progesterone and IL10 significantly increased the survival rate of xenografted hAMC-derived cardiomyocytes in the Wistar rat heart **(G). Scale bars:** 50 μm **(B,D, and E).**

they were positive for sarcomeric α-actinin, connexin 43, and cardiac troponin-I (Figure 4C and 4D; Online Figures VI and VII). Host hearts were enzymatically isolated (Figure 4E and 4F), then EGFP-positive cardiomyocytes were selected (Figure 4G and 4H). FISH analysis to detect the human-Y and the rat-X chromosome revealed that the EGFP-negative cardiomyocytes express the rat-X chromosome and no human-Y chromosome (Figure 4I and 4K), whereas the EGFP-positive cardiomyocytes express the human-Y chromosome and no rat-X chromosome (Figure 4J and 4L). FISH analysis to detect Alu,[23] which is a human-specific short interspersed repetitive element, revealed that the EGFP-negative cardiomyocytes were negative for Alu (Figure 4M and 4P), whereas the EGFP-positive cardiomyocytes were positive for Alu (Figure 4O and 4Q). From these findings, we concluded that neither cell fusion nor nuclear fusion was the major cause of generation of EGFP-positive cardiomyocytes, but that the hAMCs transdifferentiated into cardiomyocytes and were immunologically tolerated, surviving more than 80 days in situ. In some experiment non–EGFP-labeled hAMCs was transplanted into the MI zone of EGFP-transgenic mouse, and observed EGFP-negative sarcomeric α-actinin positive hAMCs derived cardiomyocytes was observed (Online Figure VIII).

Before the transplantation, HLA-G was consistently detected in hAMCs by western blot analysis, and was also detected in mesenchymal cells obtained from other placenta-related organs (Figure 5A). After transplantation, however, HLA-G was detected only inconsistently in situ. No membrane-binding isoform of HLA-G was detected in the surviving hAMC-derived cardiomyocytes in Wistar rat hearts

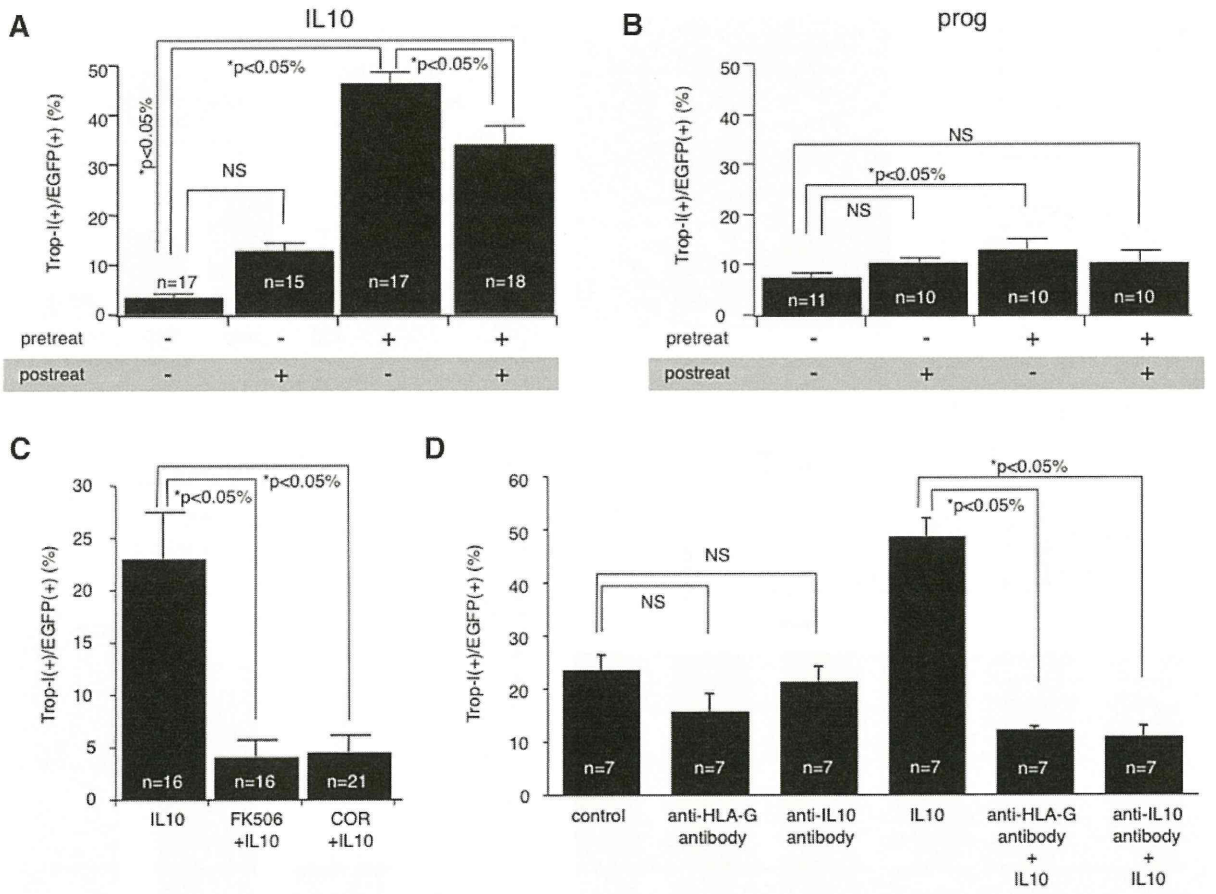

Figure 6. The effect of IL10, progesterone, and immunosuppressive agents on cardiomyogenic transdifferentiation efficiency of hAMCs. The effect of IL10 **(A)** and progesterone (prog) **(B)** on the cardiomyogenic transdifferentiation efficiency was measured. These agents were administered 2 days before the cocultivation (pretreat) and/or after the cocultivation (posttreat), and the conditions of administration are denoted below each **column**. Pretreatment with IL10 markedly increased the efficiency of cardiomyogenesis; progesterone, modestly, but significantly, increased the efficiency. **C,** IL10-induced increase in the cardiomyogenic transdifferentiation efficiency was significantly attenuated by the administration with FK506 or hydrocortisone (COR). **D,** IL10-induced increase in cardiomyogenic transdifferentiation efficiency was completely blocked by the administration of anti HLA-G antibody and anti-IL10 antibody.

(Figure 5B) by immunohistochemical analysis. There was no correlation between continuous secretion of the soluble HLA-G in the sera (ELISA) and survival of hAMC-derived cardiomyocytes (Figure 5C). On the other hand, adjacent to the surviving hAMC-derived cardiomyocytes, FOXP3-positive regulatory T cells were constantly detected by immunohistochemistry (Figure 5D and 5E; Online Figure IX), whereas they were not detected in control myocardium. The effects of IL10 or progesterone on the HLA-G expression in hAMCs were examined. Western blot analysis showed that IL10 and progesterone increased the HLA-G expression (Figure 5F). Concordantly, pretreatment with IL10 or progesterone before the hAMC transplantation significantly increased the incidence of survival of EGFP-positive hAMC-derived cardiomyocytes in vivo (Figure 5G).

Furthermore, the effect of IL10 or progesterone on the cardiomyogenic transdifferentiation efficiency of hAMCs was examined. IL10 or progesterone was administrated to the hAMCs (PDs=13) before and/or after the cardiomyogenic induction, then cardiomyogenic transdifferentiation efficiency was measured (Figure 6A and 6B). Surprisingly, both IL10 and progesterone significantly improved cardiomyogenic transdifferentiation efficiency in vitro. It is notable that pretreatment with IL10 or progesterone before cardiomyogenic induction is essential for this increase in cardiomyogenic transdifferentiation efficiency. Administration of FK506 and hydrocortisone significantly attenuated the IL10-induced increase in cardiomyogenic transdifferentiation efficiency (Figure 6C) in vitro. Moreover, the effect of IL10 was completely blocked by either anti IL10 antibody or anti HLA-G antibody administration (Figure 6D).

Discussion

We isolated hAMCs in the present study. Our isolated hAMCs can be transdifferentiated into cardiomyocytes in vitro and vivo, without using any epigenetic agent or gene transfer. The cardiomyogenic transdifferentiation efficiency of hAMCs was significantly higher than that of marrow-derived mesenchymal stem cells. Furthermore, xenografted hAMCs transdifferentiated into cardiomyocytes and survived more than 2 weeks (observed up to 80 days; Online Figure V); this suggests hAMCs were tolerated in situ. Immunologic

tolerance and cardiomyogenic transdifferentiation of hAMCs were significantly increased by pretreatment with IL10 or progesterone. From these findings, we concluded hAMCs can be a ready-to-use allograftable cellular source for cardiac stem cell therapy.

Highly Cardiomyogenic Transdifferentiation of hAMCs

The cardiomyogenic transdifferentiation efficiency of hAMCs at PD4 was calculated as 33%, which is higher than that of PCPCs.[9] Moreover, hAMCs express OCT-4 and SSEA4, a stem cell marker, and have a potential to differentiate into endodermal, mesodermal, and ectodermal lineage (Online Figure X) to some extent. These findings indicate that hAMCs have a ability to transdifferentiate into cells of various organs in comparison to other human somatic stem cells. This potential may contribute to the high cardiomyogenic transdifferentiation ability of hAMCs. The surface marker analysis clearly showed hAMCs have a mesenchymal phenotype. Despite a lack of expression of Nkx 2.5, a cardiac homeobox gene for cardiac differentiation, several cardiomyocyte-specific genes were expressed at the default state of hAMCs. Histological and physiological examinations revealed that hAMCs transdifferentiated into matured and physiologically functioning cardiomyocytes in vitro and in vivo. Moreover, transplantation of hAMCs improved cardiac functions and reduced the area of MI in vivo. The hAMCs-derived cardiomyocytes may play a role in improvement of cardiac function; however, antiapoptotic effect[24] of hAMCs may play a significant role in the present study. In the present study, neovascularization[25,26] may not play a major role in the improvement because hAMCs did not affect the capillary density (Online Figure XI).

Evidence of Tolerance

Tolerance is extremely important for clinical application of hAMCs, because we can use enormous numbers of hAMCs obtained from every delivery without establishing a stem cell bank system to match host and donor HLA-types. It is also notable that massive numbers of hAMC-derived cardiomyocytes were tolerated and survived in the xenografted host heart without using an immunosuppressant.

It is common that either allografted or xenografted cells could not survive for more than 2 weeks in the immunocompetent hosts because of rejection by the host's immune system. Therefore, massive survival of transdifferentiated EGFP-positive hAMCs in the Wistar rat heart for more than 2 weeks strongly suggests tolerance. Two independent FISH analysis clearly support the conclusion that the isolated EGFP-positive cardiomyocytes are of hAMC origin (have human nuclei) and are evidence of tolerance in the host heart.

The Mechanism of Tolerance

Because placenta and amniotic membrane are known to play an important role in avoiding maternal immunologic rejection against the fetal tissue bearing paternal alloantigens during normal gestation, it may be possible that the engrafted hAMCs were immunologically tolerated in the host heart. In comparison to other mesenchymal cells, hAMCs express lower MHC antigens; this might have importance for inducing tolerance in the host, because xenografted MMCs,[7] which express HLA-G and HLA-ABC, were completely rejected (n=4, data not shown). The expression pattern of HLAs suggests that hAMCs are resistant to MHC-dependent rejection mediated by T cell immune systems, but are known to be rejected by substitutive mechanisms. Cells that do not express the MHC molecule may be recognized as missing self cells, and may be attacked by natural killer cells.[27]

The nonclassic MHC class I antigen HLA-G expressed on the extravillous cytotrophoblast cells at the fetomaternal interface, is thought to play a major role in protecting the fetus from maternal rejection by natural killer cells.[28] Furthermore, HLA-G blocks the immunologic response of natural killer cells[29] and induces regulatory T cells,[30] which play an important role in immunologic tolerance.[31–33] In the present study, despite the fact that hAMCs expressed HLA-G in vitro, there is no correlation between the survival of EGFP-positive cardiomyocytes and continuous secretion of sHLA-G/HLA-G in Wistar rats in vivo. From these findings we speculated that HLA-G might play a role in the initial process of tolerance; however, it may not play a major role in tolerance maintenance.

A previous report showed that HLA-G–induced regulatory T cells, defined as FOXP3 positive lymphocytes,[30] also play a significant role in tolerance maintenance. Emergence and mobilization of FOXP3-positive lymphocytes beside the survived EGFP-positive cardiomyocytes in the present study strongly suggest that the regulatory T cell also plays an important role in maintenance of immunologic tolerance. However, it is difficult to verify this hypothesis by observing survival of hAMC-derived cardiomyocytes to evaluate tolerance, because the blockade of HLA-G must inhibit cardiomyogenic transdifferentiation efficiency in vivo. Because both tolerance and transdifferentiation efficiency increase the number of hAMC-derived cardiomyocytes in vivo, it is difficult to demonstrate direct evidence for the role of HLA-G in tolerance by simply determining the number of hAMC-derived cardiomyocytes. Further experimentation should be performed.

IL10, known as an immunosuppressive cytokine, is produced by regulatory T cells and type 2 helper T cells.[34,35] Progesterone and IL10, which are known to play an important role in causing fetomaternal immunologic tolerance and maintenance of normal pregnancy,[32,36,37] dramatically increase the HLA-G secretion from hAMCs. Concordant with the degree of HLA-G expression, the pretreatment of hAMCs with IL10 significantly increased the survival rate of EGFP-positive cardiomyocytes in Wistar rat hearts. This also suggested the major role of HLA-G in immunologic tolerance in the present study.

Relation Between the Immunologic Reaction and Cardiomyogenic Transdifferentiation of hAMCs

The mechanism of cardiac transdifferentiation of hAMCs is still undetermined; however, it is notable that the immunosuppressive cytokine IL10, or progesterone, dramatically increased the cardiomyogenic transdifferentiation efficiency, whereas FK506 or hydrocortisone attenuated the efficiency. This finding was also associated with the fact that mesenchymal cells, having significant cardiomyogenic transdifferentiation ability, can be obtained from gestation-related organs, ie, umbilical cord blood,[8] uterine endometrium,[7] menstrual

blood,[7] placenta,[9] and amniotic membrane, and gestation is one of the best circumstances for immunologic tolerance.

Because pretreatment of hAMCs with IL10 before coculture was essential for the increase in cardiomyogenic transdifferentiation efficiency, the main effect of IL10 should be on the hAMCs. IL10 increased the HLA-G expression in hAMCs and the effect on the cardiomyogenic transdifferentiation was blocked by the administration of anti-IL10 antibody or anti–HLA-G antibody, suggesting IL10-induced HLA-G expression plays a pivotal role in increasing the efficiency of cardiomyogenic transdifferentiation. Administration of IL10 without coculture did not cause any cardiomyogenic transdifferentiation in vitro; therefore, the HLA-G–dependent increase in the cardiomyogenesis required the feeder cardiomyocyte culture. The fact that treatment with FK506 or hydrocortisone significantly attenuated the cardiomyogenic transdifferentiation efficiency strongly suggests that inflammation activity in feeder cultures plays a pivotal role in cardiomyogenic transdifferentiation of mesenchymal cells. The different responses in cardiomyogenesis by the same immunologic suppressant may be caused by the different mechanisms of the agents. FK506 and hydrocortisone suppress every immunologic process, whereas HLA-G is known to suppress natural killer cells[38] and activate immunosuppressive regulatory T cells.[30] The process of HLA-G–dependent induction of immunologic reaction may be a clue to understanding cardiomyogenic transdifferentiation of hAMCs in vitro and vivo. Further experimentation should be performed.

Clinical Contributions

Amniotic membrane can be obtained at every delivery. Because it is usually considered as medical waste, it is an easily accessible cellular source without ethical problems. Our established hAMCs can transdifferentiate into cells of various organs, especially into functioning cardiomyocytes. For safety concerns (ie, neoplasm formation), hAMCs may be superior to the iPS cells, because hAMCs do not require any genetic or epigenetic modifications. We did not observed tumor and/or teratoma formation (also see the Online Data Supplement) in the present study. The noncardiomyogenic transdifferentiation efficiency of hAMCs in vitro was low and hAMCs are subject to contact inhibition and anchorage dependence during multiplication. These cellular characteristics might contribute to the low incidence of neoplasm formation of hAMCs. Regarding immunologic rejection, hAMCs may have an equivalent potential to iPS cells. Because pretreatment with IL10 significantly increased cardiomyogenic transdifferentiation efficiency and immunologic tolerance, we may be able to use hAMCs as an allograftable stem cell source for cardiac stem cell therapy. Compared to the other human mesenchymal cells,[6–9] only hAMCs can be immunologically tolerated and transdifferentiate into cardiomyocytes in vivo. Because they can be used as an allograft, hAMCs can be used for clinical patients immediately without establishing a stem cell bank system.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- The amniotic membrane is believed to play an important role in fetomaternal tolerance during normal pregnancy.
- Cardiomyogenic transdifferentiation efficiency of human marrow-derived mesenchymal stem cells are extremely low (≈0.3%).
- There is no proven, ready-to-use cellular source for cardiac stem cell–based therapy.

What New Information Does This Article Contribute?

- Cardiomyogenic transdifferentiation efficiency of human amniotic membrane–derived mesenchymal stem cells is ≈33%.
- Amniotic membrane, which can be obtained from every delivery, can be a good, ready-to-use cellular source for cardiac stem cell–based therapy in other patients.
- It is possible that these cells will be immune-privileged.
- Immunologic tolerance and cardiomyogenic transdifferentiation of human amniotic membrane–derived mesenchymal stem cells are significantly increased by pretreatment with IL10 or progesterone.

There is no definitive ready-to-use cellular source for cardiac stem cell therapy. Our present study showed that xenografted human amniotic membrane–derived mesenchymal stem cells (hAMCs) transdifferentiated into cardiomyocytes and were tolerated >80 days in situ. This finding suggested that hAMCs could be an ideal allograftable cardiac stem cell source. Because hAMCs do not cause immunologic rejection in the host, we do not need to adjust donor–host immunologic matching of human leukocyte antigens. Therefore, we can use freshly isolated hAMCs immediately in allogenic combination without establishing stem cell bank systems. Moreover, the precise mechanism of cardiomyogenic transdifferentiation is unclear. We showed close relationships between the mechanisms of cardiomyogenic transdifferentiation and the process of causing immunologic tolerance. This may be a key to understanding of the mechanism of cardiomyogenic transdifferentiation. Consequently, we may be able to manipulate the cardiomyogenic transdifferentiation efficiency. Our findings are important to advance clinical cardiac stem cell therapy.