

の開発段階に応じて設定される相談、タイプCはそれ以外の相談である。タイプBにはPre-IND、第1相終了時、第2相終了時、販売申請前の4種類がある。Pre-IND相談は非公式なもので、INDの内容を簡略化した資料をFDAに提出、IND本申請におけるポイント、質問事項、問題点を相談できる制度である。第1相終了時相談以降の相談はデータを検討して次の開発ステップの計画についての相談・合意のために実施される公式なものである。また、がん原性試験、最終製品の安定性試験、ないし主要な第3相試験のプロトコルについて試験開始前に相談できる制度として特別プロトコル評価 (special protocol assessment; SPA) がある⁵⁾。SPAでは申請者と当局との間で合意に至った事項等が明記された文書が作成される。SPAで合意されたプロトコルは販売承認審査で効力がある。

医療機器の開発者とFDAとがIDE前に行う相談にはPre-IDE非公式相談、決定相談、合意相談の3種類がある。Pre-IDE非公式相談の目的は、製品を上市するためのプランを共同で編み出すことであり、申請書に盛り込む非臨床データ、臨床試験プロトコル、評価指標・エンドポイント、手続き、審査スケジュールなどが議題となる。決定相談は、製品の有効性を示すためにはどういった科学的根拠が必要かを当局が決定し、申請者に示すために実施される。合意相談は、臨床プロトコルなど、開発の主要な点について申請者と当局とが合意するために実施される。

3) 臨床試験と未承認製品へのアクセス

米国では製品化を目的とするかどうかに関わらず、未承認351HCT/Pの臨床試験を行う場合には、FDAに申請して承認を得る必要がある。対象となる351HCT/P

Pが生物製剤の場合にはこれをIND (Investigational New Drug Application)、医療機器の場合にはIDE (Investigational Device Exemption Application) という。いずれも原則として初回審査期間は30日であり、GCPの遵守が原則である。

生物製剤の場合、企業による製品開発のための臨床試験の申請をCommercial IND、医師や大学の研究者が研究目的で行う臨床試験の申請をNon-commercial INDと呼ぶ。Non-commercial INDには3種類あり、通常のもはInvestigator IND、特定の個人患者への人道的使用のためのINDはSingle Patient IND、重篤で生命を脅かす疾患の治療のために未承認生物製剤を臨床試験プロトコル外で例外的に使用する際のINDはTreatment INDと呼ばれる。Single Patient INDのうち、特に緊急時のものはEmergency INDとも呼ばれる。Commercial INDとInvestigator INDとでは、審査要件に原則的に違いはない。Single Patient INDとTreatment INDとは、緊急性およびGCP準拠の臨床試験データを前提とするかどうかなどで違いがあるものの、ともに未承認生物製剤の例外的・人道的使用のための道筋として設けられている。

米国では医療機器は患者や使用者に影響を及ぼすリスクの程度によってクラスⅠ、Ⅱ、Ⅲに分かれており、351HCT/Pの場合、そのほとんどが最も規制が厳しいクラスⅢに属すると考えられ、IDEの申請の必要がある。生物製剤と同様、企業が主体の場合はCommercial IDE、研究者主導の場合はInvestigator IDEと呼ばれる。

ただし、治療法の無い重篤ないし致死的な状態にある患者の治療のため、販売未承認の医療機器の使

表1 351HCT/PとはならないHCT/Pの要件

1. HCT/Pの加工が最小限 (minimal manipulation) **
2. HCT/Pが、細胞・組織の採取部位と同等な部位への適用 (homologous use) にのみ限定される場合で、そのことが表示、宣伝等に反映されている
3. 製造工程に他の物質 (水、クリスタロイド、滅菌剤、保存剤、または保管剤を除く) と細胞または組織との複合体化が含まれず、かつ水、クリスタロイド、滅菌剤、保存剤、または保管剤の添加によって当該HCT/Pに関して新たな臨床上の安全の上での懸念を生じない場合
4. 以下の何れかに該当する場合
(1) HCT/Pに全身的な作用がなく、その主たる機能として生細胞の代謝活性に依存することがない
(2) HCT/Pに全身的影響がある、またはその主たる機能として生細胞の代謝活性に依存することがあり、かつ以下の何れかに該当する場合
① 自己への使用を目的とする
② 一親等または二親等の血縁関係の同種のための使用である
③ 生殖目的の使用である

* 上の1. ~ 4.のすべてが該当する場合以外はHCT/Pは351HCT/Pと見なされる。

** 最低限の処理 (minimal manipulation) の要件は文献3参照

用が必要となる場合もある。こうした状況にある患者・医師は、FDAが規定する4種の道筋、すなわち Emergency Use、Compassionate Use、Treatment Use、Continued Accessのいずれかの道筋を通じ、臨床試験中の医療機器にアクセスすることができる。Emergency Useは臨床試験に参加していない医師が試験中の医療機器を緊急に使う必要が生じた場合、Compassionate Useは臨床試験の基準に合致しない患者だが担当医師がその患者の疾患・病態の治療・診断に有益だと判断した場合、Treatment Useは有望な臨床試験データが出た際に重篤ないし生命の危機にある患者を追加する場合、Continued Accessは臨床試験の終了後かつ販売承認前に当該機器が公衆衛生上必要となる場合の道筋である。また、米国内の患者数が少ない（年4千人以下）場合で、他に有効な製品がない病態の治療・診断に用いるための医療機器は人道用機器（Humanitarian Use Device; HUD）と呼ばれ、この場合は臨床試験以外のアクセスを許容するのではなく、想定されるベネフィットが病態のリスクを上回ることと製品自体の安全性が示されれば有効性データがなくとも販売承認を与えるという措置が取られている。これを人道機器免除（Humanitarian Device Exemption; HDE）という。

4—EUの規制

欧州連合（EU）では細胞・組織加工製品は、製品中に含まれる細胞の作用様式に基づき体細胞治療薬（薬理的・免疫学的又は代謝的機能）または組織工学製品（ヒト組織の再生・修復又は置換）に分類される（表2）。

従来、体細胞治療薬は遺伝子治療薬とともにATMP（advanced therapy medicinal product）という医薬品の一類型に分類されていたが、2008年12月の制度改正により組織工学製品もATMPとして医薬品の規制を受けることになった⁶⁾。これと同時に、ATMPの販売承認については加盟国における審査を経ずに欧州医薬品庁（EMA）による中央審査によって行われるようになっており、制度改正からこれまでに培養軟骨製品1品目がATMPとして販売承認を受けている。

1) EMA・EU加盟国規制当局による開発支援

EMAはEUの医薬品産業の強化に必要な新技術の開発支援に積極的な姿勢を示しており、医薬品の製品開発に関する科学的助言やプロトコル支援を開発者に提供している。ATMPの開発者は中小のベンチャー企業等（small and medium-sized enterprise; SME）が多いことから、SMEがATMPについての科学的助言を必要とする場合、通常の手数料の90%割引で相談に応じている。相談者がSMEで、対象となるATMPが公衆衛生上の特別な利益となることが証明できる場合には、更なる割引が考慮される。なお、それ以外の開発者でも対象品目がATMPならば通常の65%割引で相談に応じている。また、オーファン医薬品の場合のプロトコル支援は無料である。ATMPに関する場合には、上記相談以外に、より非公式な制度として技術革新タスクフォース（Innovation Task Force; ITF）との相談も利用可能である。ITFはEMA内の多部署から成るグループで、先端的治療・技術について規制面での問題点を討議することを目的としている。この制度はITFから助言を受けるというよりも意

表2 EUにおける「体細胞治療薬」と「組織工学製品」の定義

	体細胞治療薬	組織工学製品
1. 使用目的	製品に含まれる細胞・組織の薬理的、免疫学的または代謝的作用を通じた疾患の治療、予防または診断	ヒト組織の再生、修復または置換
2. 以下のいずれかに該当する細胞・組織を含む（又はそうした細胞・組織で構成される）		
実質的加工*	使用目的に適うように生物学的性質、生理学的機能または構造上の特性を変化させる実質的加工を施された細胞・組織	
細胞・組織の機能	ドナーの体内での本来の機能と同じ機能を患者の体内でも果たすことを意図して利用するのではない細胞・組織	

* 実質的加工に含まれない加工の具体例については文献6のAnnex 1参照

見交換の意味合いが強い。ITFとの相談は無料であるが論議内容の法的拘束力はない。さらにこれらの制度とは別にEMAは、開発者の品目がATMPに該当するか否かの助言を無料で行うとともに、大企業への技術移転促進を意図して、SMEの非臨床試験・品質試験のデータの科学性に関する審査とその暫定認証（公式には治験届や承認申請での効力はない）を臨床試験開始・承認申請の有無に拘わらず随時、無料で行っている。またEMAは、2009年6月に培養軟骨製品の販売承認が下りると即座にその審査経験をもとに培養軟骨製品の承認審査における留意点をまとめた文書を公表したり⁷⁾、研究開発が進むiPS細胞等の多能性幹細胞を加工したATMPに関する特別な留意点をまとめた文書を公表したりする⁸⁾など、製品開発の道筋を示す活動も精力的に行っている。

加盟各国の規制当局もそれぞれ独自に、商業目的・非商業目的に拘わらず臨床試験開始前の開発早期から相談を安価ないし無料で受け付けるなど、ATMPの開発支援に積極的である。例えばイギリスでは、国内でATMPの開発を目指す場合には、医薬品庁（MHRA）と面談して科学的助言を得ることができる。相談内容としては例えば、品質、非臨床試験、臨床試験、市販後調査、規制面などが挙げられる。なお、MHRAからの科学的助言は、将来の製品の諸申請とは法的には繋がりをもたないが、face-to-faceの相談であることとEMAよりも安価であるという利点がある。ドイツでは、ATMPを管轄するポールエールリッヒ研究所（PEI）に、ATMPの開発推進を目的としたイノベーション・オフィスが設置されている。イノベーション・オフィスは大学や臨床研究グループ、中小企業および独立事業部門などの民間セクターを対象に、医療製品開発の非常に初期の研究段階（製品分類、リスク分析の方法論、臨床試験前の相談）から国内製造承認、臨床試験申請やEMAへの販売承認申請まで、ATMPの開発を包括的に支援している。フランスでATMPを管轄する保健製品衛生安全庁（AFSSAPS）も、ATMPの開発を支援する目的で開発者との相談制度を設け、科学的見地からの助言を提供している。相談は、製品開発のどの段階でも構わない。相談相手としてAFSSAPS内の審査員だけでなく関連分野の外部専門家が選ばれることもあり、製品の品質、安全性、製造、臨床試験等について無料で討議される。

2) 臨床試験と未承認製品へのアクセス

EMAはあくまで販売承認審査を行う機関であり、EUでは臨床試験の開始・実施に関する手続きは加盟国の管轄となっている。EU各国におけるATMPの臨床試験は、商業目的か非営利目的かに拘わらず、臨床試験申請の審査はEMAのガイドラインに従って行

われる。臨床試験におけるGTP・GMPについては、EMA販売承認後のGTP・GMPと比較した場合、状況によってはデータの例数が少なくとも許容されるなどの量的緩和はありうるが、質的緩和は原則的にはないとされている。

臨床試験以外に、患者がEU未承認のATMPにアクセスできる道筋としては大きく分けて、Reg (EC) No 1394/2007 Article 28に基づく「病院免除」(Hospital Exemption)、Reg (EC) No 726/2004 Article 83 (1)に基づく「人道的使用」(Compassionate Use)、Dir 2001/83/EC Article 5(1)に基づく「特別免除」(Special Exemption)の3種類がある。「病院免除」は、販売未承認のATMPについて、①特定の一患者向けの特注品の処方箋に従って、②固有の品質基準に基づき、③非反復的に製造され、④医療従事者の職務責任の下、⑤同一加盟国内で、⑥単一病院において使用される、という条件を満たす場合にはEMAの中央審査の対象とならないという規定である。ただし、この枠組みにおいても、製造・使用国における製造・品質に関する承認、ファーマコビジランス、トレーサビリティの確保が要求される点で注意を要する。「人道的使用」は、慢性的もしくは重度の衰弱をもたらす疾患や生命に関わると考えられる疾患を持ち、かつ既存の製品では十分な治療ができない患者群に対して未承認医療製品を使用することとされる。このルートの適用を受けることが可能な製品は、EMAへの販売承認申請予定の品目もしくは臨床試験中の品目に限る。「特別免除」は、「患者からの全くの自発的な要望に応じて供される医療製品で、医療資格者の仕様に基づいて調製され、医療資格者が自らの直接的かつ個人的な責任において使用するためのもの」については販売承認を受ける必要がないという制度であり、個人向けの人道的使用と考えることも出来る。

5—おわりに

欧米の細胞・組織加工製品の開発に関する環境は、資金面、インフラ面の他、上に挙げたように規制に関しても日本とは大きく異なっている部分がある。商業目的か非商業目的かに拘わらずICH-GCPが要求される点などは、日本の再生医療・細胞治療の開発環境よりも厳しい公衆衛生的観点からの規制がかかっていると言える。しかし彼らは同時に、患者側の観点にも配慮し、重篤ないし生命の危機にある患者が未承認の新規製品にアクセスすることを可能とし、かつその使用状況を監視できる様々な仕組みを設け、「公衆衛生」と「患者」の2つの観点との間の微妙なバランスを取ることに腐心している。我が国の規制ではこのバランスはどう保たれてきたのだろうか、ということは自問する必要があるかもしれない。

開発環境に差があるとしても、細胞・組織加工製品を効率的・効果的・合理的に実用化するためには、必要な技術的要件や方策を、出口である行政側がガイドラインや相談制度などを通じて開発早期から提示し、研究者・開発企業・規制側が認識を共有することが不可欠であることは論を待たない。ただ、欧米では非商業的臨床試験に対しても規制当局の相談窓口が開かれているのに対し、日本の「臨床研究」では開発早期には製品化・実用化に向けた規制当局との「治験相談」ができないという点が従来問題とされてきた。先ごろ（平成23年2月）開催された厚生労働省「再生医療における制度的枠組みに関する検討会」において、確認申請制度を、新たな薬事戦略相談に代替すべきだとの提言がなされた。確認申請は細胞・組織加工製品等の治験開始前にその品質・安全性の確認を行うための制度であるが、現在構想されている薬事戦略相談は治験と臨床研究の区別なく細胞・組織加工製品等の製品化に関して品質・安全性のみならず臨床プロトコルまで含めて議論を行う制度とされており、今後の展開が期待される。その際にも今回紹介した欧米の規制や実用化促進策は大いに参考となるものと考えられる。

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再生医療・細胞治療の規制に関する国際動向

Regulation of Cell Therapy Products and Tissue Engineered Products in EU and USA

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1 細胞・組織加工製品

治療法に乏しく、重篤・致命的ないしQOLを著しく損なう疾病・損傷に対する活路として、再生医療や細胞治療には非常に大きな期待が集まっている。これらの先進的な医療に用いることを目的として加工（培養・活性化・足場との複合化等）を施された細胞や組織、あるいは加工された細胞・組織を含む製品は「細胞・組織加工製品」（細胞・組織加工医薬品ないし細胞・組織加工医療機器）と呼ばれ、その開発は世界的にも熾烈な競争が展開している。ただし細胞・組織加工製品は、細胞という動的で複雑な成分を含むと同時に、その臨床応用に関して限られた経験と知識しか存在しないため、明確な科学的根拠に基づいた品質や安全性等の確保が課題となっており、比較的進んでいると言われる欧米においても、当局は実用化を促進するための試行を繰り返しながら規制の枠組みの整備を進めている。

2 規制の原則

欧米における細胞・組織加工製品の規制の原則は「リスクベースアプローチ」と呼ばれ、目的とする製品の性質に固有、かつその品質・安全性・有効性に関連するリスクの分析をベースにし、その影響の度合いを科学的に評価することにより規制や開発の方針・内容を定めるアプローチ方法が採られている。日本では、細胞・組織加工製品を医薬品・医療機器として開発するために薬事法に則って実施される「治験」と、細胞・組織加工製品を

用いた治療法の開発を目的として医療法・医師法のもとで行われる「臨床研究」という異なる規制の枠組みが存在するが、欧米ではリスクベースアプローチの原則に基づき、商業目的か非商業目的かに拘わらず、原則的には同一の規制がかかる。即ち、大学病院等による非商業目的の「臨床研究」においても国への臨床試験申請並びにICH-GCP準拠が要求される点で日本よりも厳しい制度となっている。それでも開発が進む理由としては、研究資金・臨床試験支援体制などが充実している他、規制当局等の専門家が開発早期から開発者と情報を共有し、製品の目的に沿った柔軟な対応が可能となっていることなどが挙げられる。

3 EUの規制

欧州連合（EU）では細胞・組織加工製品は、製品に含まれる細胞の作用様式に基づき体細胞治療薬（薬理的・免疫学的又は代謝的機能）または組織工学製品（ヒト組織の再生・修復又は置換）に分類される。従来、体細胞治療薬は遺伝子治療薬とともにATMP（advanced therapy medicinal product）という医薬品の一類型に分類されていたが、2008年12月の制度改正により組織工学製品もATMPとして医薬品の規制を受けることになった。これと同時に、ATMPの販売承認については加盟国における審査を経ずに欧州医薬品庁（EMA）による中央審査によって行われるようになっており、制度改正からこれまでに培養軟骨製品1品目がATMPとして販売承認を受けている。なお制度改正以前に販売承認を

受けた製品については、新審査方式での販売承認を再取得しなければ、遺伝子治療薬・体細胞治療薬の場合には2011年末、組織工学製品の場合には2012年末に販売承認が取り消されることになっている。EMAはEUの医薬品産業の強化に必要な新技術の開発支援に積極的な姿勢を示しており、開発者に対するATMPに関する科学的助言や、中小開発企業の非臨床・品質データの暫定認証を行ったりすると同時に、研究開発が進むiPS細胞等の多能性幹細胞を加工したATMPに関する特別な留意点をまとめた文書を公表するなどしている。なお、EMAはあくまで販売承認審査を行う機関であり、臨床試験の開始・実施に関する手続きは加盟国の管轄となっている。加盟各国の規制当局もそれぞれ独自に、商業目的・非商業目的に拘わらず臨床試験開始前の開発早期から相談を安価ないし無料で受け付けるなど、ATMPの開発支援に積極的である。

4 米国の規制

米国ではヒト細胞・組織を利用した製品および遺伝子治療薬はHCT/P (human cell, tissue, or cellular/tissue-based product) と総称される。中でも細胞に一定以上の加工を施したものや遺伝子治療薬などは、州を越えて流通させる場合には公衆衛生サービス法351条により食品医薬品局 (FDA) の販売承認が必要とされ、351HCT/Pと呼ばれる。EUとは異なり、351HCT/Pはその主な作用様式が細胞・組織の生化学的・免疫学的又は代謝的機能に基づく場合には生物製剤、細胞・組織の物理的又は構造的機能の場合には医療機器としての規制を受ける。これまでに2品目が生物製剤、5品目が医療機器として販売承認を受けている。EU同様、製品化を目的とするかどうかに関わらず、351HCT/Pの臨床試験を行う場合には、FDAに申請して承認を得る必要がある。近年の351HCT/Pの臨床試験申請の過半数は大学などによる非商業目的(研究目的)のものであるが、FDAは、生物製剤でも医療機器でも、商業目的・非商業目的に拘わらず臨床試験の申請前に非公式な相談を無料で行うほか、各種の開発段階において相談制度を設けて351HCT/Pの開発を支援している

5 おわりに

欧米の細胞・組織加工製品の開発に関する環境は、資金面、インフラ面の他、上に挙げたように規制に関しても日本とは大きく異なっている部分がある。ただし、細胞・組織加工製品を効率的・効果的・合理的に実用化するためには、必要な技術的要件や方策を、出口である行政側がガイドラインや相談制度などを通じて開発早期から提示し、研究者・開発企業・規制側が認識を共有することが不可欠であることは共通である。我が国における先端医療の実用化促進や規制の国際協調のためにも彼らの規制および促進策は参考とすべきものと考えられる。

DNA microarray profiling identified a new role of growth hormone in vascular remodeling of rat ductus arteriosus

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Abstract The ductus arteriosus (DA), a fetal arterial connection between the pulmonary artery and the aorta, has a character distinct from the adjacent arteries. We compared the transcriptional profiles of the DA and the aorta of Wistar rat fetuses on embryonic day 19 (preterm) and day 21 (near-term) using DNA microarray analyses. We found that 39 genes were expressed 2.5-fold greater in the DA than in the aorta. Growth hormone (GH) receptor (GHR) exhibited the most significant difference in expression. Then, we found that GH significantly promoted migration of DA smooth muscle cells (SMCs), thus enhancing the intimal cushion formation of the DA explants. GH also regulated the expression of cytoskeletal genes in DA SMCs, which may retain a synthetic phenotype in the smooth muscle-specific cytoskeletal genes. Thus, the

present study revealed that GH-GHR signal played a role in the vascular remodeling of the DA.

Keywords Growth hormone · Gene expression · Vascular remodeling · Premature infant · Congenital heart disease

Introduction

The ductus arteriosus (DA), a fetal arterial connection between the pulmonary artery and the descending aorta, is essential to fetal life. The morphology and function of the DA dramatically change during development [1]. In particular, during late gestation, the deposition of extracellular matrix in the subendothelium is increased, and the smooth muscle cells (SMCs) of the media migrate into this region, resulting in intimal thickening [2]. This vascular remodeling of the DA is essential for its postnatal closure and is not observed in adjacent arteries. Thus, the DA has distinct characteristic features that differ from those of the adjacent arteries (the aorta and pulmonary arteries). This characteristic of the DA is largely dependent on the expression of the distinct subsets of genes involved in the developmental vascular remodeling that occurs during gestation. To understand the precise transcriptional network in the DA, genome-wide analysis is a powerful approach that can be utilized. In this context, several studies, including ours, have been carried out to identify the effects of oxygen [3] or maternal administration of vitamin A [4] on the transcriptional profiles of the DA. Although the study by Costa et al. [3] is the only one that demonstrated the characteristic differences in the transcriptional profiles between rat DA and the aorta of premature fetuses and neonates, they analyzed their transcriptional profiles on embryonic day 19 (e19) only; they did not examine the changes during later gestation.

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Because the morphological and physiological characteristics of the DA differ significantly between premature and mature fetuses [1], it is of great interest to investigate the transcriptional profiles of the DA and the adjacent aorta in the remodeling process that occurs during late gestation.

Materials and methods

Tissue collection for DNA microarray and quantitative reverse transcription polymerase chain reaction analyses

Pooled tissues from the DA or the aorta were obtained from Wistar rat embryos on e19 ($n \geq 120$) and e21 ($n \geq 120$). Reverse transcription polymerase chain reaction (RT-PCR) analysis was performed as described previously [2]. The information on PCR primers for RT-PCR analyses is provided in Supplemental data 1.

Total RNA preparation and DNA microarray analysis

Total RNA preparation and DNA microarray analysis were performed as described previously [4]. Briefly, total RNA was converted to biotin-labeled cRNA that was hybridized to rat genome U34A GeneChip DNA microarray (Affymetrix, Santa Clara, CA). The hybridization experiments were performed in duplicate and the intensities were averaged. If the difference in the signal intensities of a given sequence tag was equal to the cutoff (≥ 2.5 -fold) or more, and if the "Comparison Analysis" of the Microarray Suite Software indicated "increased" or "decreased" with the ≥ 2.5 -fold difference at any developmental stage, that sequence tag was considered to exhibit a significant difference between the DA and the aorta.

Primary culture of rat DA SMCs

Vascular SMCs in primary culture were obtained from the DAs of Wistar rat embryos at e21. The tissues were minced and transferred to a 1.5-ml centrifuge tube that contained 800 μ l of collagenase-dispase enzyme mixture [1.5 mg/ml collagenase-dispase (Roche), 0.5 mg/ml elastase type II-A (Sigma Immunochemicals, St. Louis, MO), 1 mg/ml trypsin inhibitor type I-S (Sigma), and 2 mg/ml bovine serum albumin fraction V (Sigma) in Hanks' balanced salt solution (Sigma)]. The digestion was carried out at 37°C for 15–20 min. Then cell suspensions were centrifuged, and the medium was changed to the collagenase II enzyme mixture [1 mg/ml collagenase II (Worthington), 0.3 mg/ml trypsin inhibitor type I-S, and 2 mg/ml bovine serum albumin fraction V in Hanks' balanced salt solution]. After 12 min of incubation at 37°C, cell suspensions were

transferred to growth medium in 35-mm poly-L-lysine (Sigma)-coated dishes in a moist tissue culture incubator at 37°C in 5% CO₂, 95% ambient mixed air. The growth medium contained Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution (Sigma). The confluent cells were used at passages 4–6.

SMC migration assay

The migration assay was performed using 24-well Transwell culture inserts with polycarbonate membranes (8- μ m pores; Corning Inc.) coated with fibronectin. The DA SMCs were harvested with trypsin-ethylenediamine tetraacetic acid (EDTA), resuspended in serum-free DMEM, and distributed at a density of 1×10^5 cells/100 μ l in the inserts. The cells were allowed to settle in serum-free DMEM for 1 h before the addition of GH (20 and 200 ng/ml) in the lower chamber. Under basal conditions, the lower chambers were filled with 600 μ l serum-free DMEM. SMCs were then allowed to migrate to the underside of the insert's membrane at 37°C/5% CO₂. At the end of the experiment, the cells were fixed in 10% buffered formalin. SMCs were stained with Cyto Quick (Muto Pure Chemicals), and cells on the upper surface of the membrane were mechanically removed with a cotton swab. Cells that migrated onto the lower surface of the membrane were manually counted from three different fields (0.5 mm²/field) under a microscope.

Cell proliferation assays

[³H]thymidine incorporation was used to measure cell proliferation in DA SMCs. The SMCs were reseeded into a 24-well culture plate at an initial density of 1×10^5 cells per well for 24 h before serum starvation with DMEM containing 0.1% FBS. Cells were then incubated with or without GH (20 and 200 ng/ml) for 24 h in the starvation medium before addition of 1 μ Ci of [*methyl*-³H]thymidine (specific activity 5 Ci/mM; Amersham International, Bucks, UK) for 4 h at 37°C. After fixation with 1.0 ml of 10% trichloroacetic acid, the cells were solubilized with 0.5 ml of 0.5 M NaOH and then neutralized with 0.25 ml of 1 N HCl. A liquid scintillation counter was used to measure [³H]thymidine incorporation. Data obtained from triplicate wells were averaged.

Quantitation of hyaluronan

The amount of hyaluronan in the cell culture supernatant was measured by a latex agglutination method based on the specific interaction of hyaluronan with the latex-labeled hyaluronan-binding protein from bovine cartilage (Fujirebio Inc.). Hyaluronan was quantified in duplicate according to

the manufacturer's instructions using 2.5- μ l aliquots of the conditioned cell culture medium using the HITACHI 7070 analysis system (Hitachi) at an 800-nm wavelength.

Organ culture

Fetal arteries including the DA and the aortic arch arteries were incubated with GH (200 ng/ml) for 72 h in serum-free DMEM as described previously [2]. Explants were then fixed in 10% buffered formalin and embedded in paraffin. The sectioned segments in the middle portion of the DA were analyzed histochemically.

Immunohistochemistry

Tissue staining and immunohistochemistry were performed as described previously [5, 6]. Mouse monoclonal anti-GHR antibody (MAB263) was purchased from Abcam (Tokyo, Japan).

Statistics

Data are presented as mean \pm standard error (SEM) of independent experiments. Statistical analysis was performed between two groups by unpaired two-tailed Student's *t* test or unpaired *t* test with Welch correction, and among multiple groups by one-way analysis of variance (ANOVA) followed by Neuman-Keuls multiple comparison test. A *p* value of <0.05 was considered significant.

Results

Genes differentially expressed between the DA and the aorta

All the microarray data in the present study were deposited at the Gene Expression Omnibus (GEO) repository (<http://www.ncbi.nlm.nih.gov/projects/geo/>; accession no. GSE3422). A total of 117 genes (142 probe sets) showed a significant difference (≥ 2.5 -fold) between the DA and the aorta at e19 or e21. Among 117 genes, 39 (43 probe sets) exhibited a DA-dominant expression pattern (Table 1), and 78 (99 probe sets) exhibited an aorta-dominant expression pattern (Table 2).

Of 39 genes in the DA-dominant expression pattern (Table 1), 34 had a known function, and 3 were homologous to known genes. Although several genes, such as prostaglandin E receptor 4 (subtype EP4) (Ptger4) and endothelin-1, are known to play an important role in the regulation of vascular tone of the DA [1], the role of most of the other genes in the DA has not been identified. We found that growth hormone (GH) receptor exhibited the

highest difference in the expression between the DA and the aorta among 39 DA-dominant genes.

Of the 39 genes, 9 encode proteins related to cytoskeleton and the extracellular matrix, including sarcomeric genes such as Myh11 (myosin heavy chain 11), Myl6 (myosin light chain, polypeptide 6, alkali, smooth muscle and non-muscle), Actg2 (actin, gamma 2), Tpm1 (tropomyosin 1, alpha), Tnn (tenascin N, predicted), and Lamb2 (laminin beta2). Three membrane ion channels, ATPase Na⁺/K⁺ transporting b1 polypeptide and potassium inwardly rectifying channel (Atp1b1), subfamily J, member 8 (Kcnj8), which is known as ATP-sensitive potassium channel K_{ATP}-1, and Ca²⁺ channel, voltage-dependent, $\alpha 2/\delta$ subunit 1 (Cacna2d1), were also strongly expressed in the DA.

We also identified 79 genes in the aorta-dominant expression pattern (Table 2). Of the 79 genes, 14 genes encode proteins related to cytoskeleton and the extracellular matrix. Cardiac sarcomeric genes such as Myh6 (myosin heavy chain, polypeptide 6), Myh7 (myosin heavy chain, polypeptide 7), Myl7 (myosin, light polypeptide 7, regulatory), Myl2 (myosin regulatory light chain 2, ventricular/cardiac muscle isoform), Actc1 (alpha, actin alpha cardiac 1), Tnnt2 (troponin T2, cardiac), Tnni3 (troponin I, cardiac), and Fn1 (fibronectin 1) were more highly expressed in the aorta than in the DA. Accordingly, there was a marked difference in the composition of the genes related to the cytoskeleton and the extracellular matrix between the DA and the aorta. Sixteen genes were expressed 2.5-fold more in the aorta at both e19 and e21 than in the DA, whereas 24 genes were expressed 2.5-fold more in the aorta than in the DA only at e21. To confirm the results of the DNA microarray, we performed RT-PCR (Supplemental data 2).

Growth hormone receptor mRNA and protein were dominantly expressed in the developing DA

As mentioned above, GH receptor (GHR) exhibited the highest difference of expression between the DA and the aorta (Fig. 1a), suggesting that GH-GHR signal plays a distinct role in the vascular remodeling of the DA from the aorta. The expression of GH mRNA was very low, and there was no difference between the DA and the aorta (Fig. 1b). Interestingly, the expression levels of insulin-like growth factor (IGF)-I and IGF-II mRNAs were higher in the aorta than in the DA, whereas the expression levels of IGF-I receptor (IGF-IR) and IGF-IIR mRNAs did not differ (Fig. 1c–f). In addition, the expression levels of IGF binding protein (IGFBP) 2 and IGFBP5 mRNAs were also higher in the aorta than in the DA at e21 and at e19, respectively (Table 2).

The expression of GHR mRNA was also confirmed by quantitative RT-PCR analyses. We found that the expression levels of GHR mRNA were higher in the rat DA than

Table 1 DA-dominant genes

	Probe set ID	RefSeq Transcript ID	Gene title	Gene symbol	Fold difference between DA and aorta	
					e19	e21
1	rc_AI104225_at	NM_017094	Growth hormone receptor	Ghr	5.0	8.7
2	Z83757mRNA_at	XM_222794	Tenascin N (predicted)	Tnn_predicted	2.3	7.4
3	L16764_s_at/// Z75029_s_at	NM_031971/// NM_212504	Heat shock 70kD protein 1A///heat shock 70kD protein 1B	Hspa1a///Hspa1b	8.6	7.1
4	rc_AA891527_at	NM_022531	Desmin	Des	1.4	6.3
5	rc_AA893846_at	NM_053591	Dipeptidase 1 (renal)	Dpep1	3.4	5.6
6	X73524_at	NM_013129	Interleukin 15	Il15	2.0	5.6
7	D28561_s_at	NM_031677	Four and a half LIM domains 2	Fhl2	3.4	4.9
8	rc_AA894200_at	XM_342032	Proprotein convertase subtilisin/kexin type 5	Pcsk5	1.4	4.8
9	AF002281_at	NM_012870	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	Tnfrsf11b	1.2	4.7
10	M22323_at	NM_013086/// NM_017334	cAMP responsive element modulator	Crem	1.8	3.5
11	U94330_at	NM_001007678	Mss4 protein	Mss4	1.5	3.1
12	rc_AA799773_at	NM_134410	Mg87 protein	Mg87	1.3	2.9
13	X82152_at	NM_001002287	MAS-related G protein-coupled receptor, member B4	Mrgprb4	2.5	2.9
14	M64711_at	NM_080698	Fibromodulin	Fmod	1.6	2.8
15	D28860_s_at	NM_012893	Actin, gamma 2	Actg2	3.7	2.8
16	U69272_g_at	NM_017099	Potassium inwardly rectifying channel, subfamily J, member 8	Kcnj8	2.8	2.8
17	rc_AA859578_at	NM_021587	Latent transforming growth factor beta binding protein 1	Ltbp1	2.0	2.7
18	rc_AA859954_at	NM_012751	Solute carrier family 2 (facilitated glucose transporter), member 4	Slc2a4	2.3	2.7
19	rc_AI014135_g_at	NM_013113	ATPase, Na ⁺ /K ⁺ transporting, beta 1 polypeptide	Atp1b1	2.1	2.7
20	rc_AI176662_s_at	NM_019131	Tropomyosin 1, alpha	Tpm1	2.3	2.7
21	AB020504_g_at	NM_032076	Prostaglandin E receptor 4 (subtype EP4)	Ptger4	2.4	2.6
22	rc_AI232078_at	NM_012827	Bone morphogenetic protein 4	Bmp4	2.8	2.6
23	U02553cnds_s_at	NM_033485	PRKC, apoptosis, WT1, regulator	Pawr	1.4	2.6
24	X63253cnds_s_at	NM_080902	Hypoxia induced gene 1	Hig1	1.7	2.6
25	M60921_g_at	XR_086177	PMF32 protein (predicted)	Pmf31	2.8	2.5
26	S66024_at	XM_343144	Myosin, light polypeptide 6, alkali, smooth muscle and non-muscle (predicted)	Myl6_predicted	2.7	2.5
27	M86621_at	NM_012887	Thymopoietin	Tmpo	1.2	2.5
28	X54686cnds_at/// rc_AA891041_at	XM_573030	Myosin heavy chain 11	Myh11	3.7	2.3
29	U17254_g_at	NM_012974	Laminin, beta 2	Lamb2	2.5	2.3
30	Z22607_at	NM_019620	Zinc finger protein 386 (Kruppel-like)	Znf386	7.5	2.2
31	rc_AA891422_at	NM_053650	PDZ and LIM domain 3	Pdlim3	2.6	1.8
32	rc_AI144767_s_at/// rc_AA875132_at	NM_024162	Fatty acid binding protein 3	Fabp3	3.5	1.7
33	M63656_s_at	NM_012919	Calcium channel, voltage-dependent, alpha2/delta subunit 1	Cacna2d1	2.7	1.6
34	rc_AI014163_at	NM_012548	Endothelin 1	Edn1	2.8	1.4
35	rc_AI639161_at	XM_346029	Similar to KIAA1411 protein (predicted)	RGD1304927_predicted	2.7	1.2
36	rc_AA866345_at	NM_012531	Catechol-O-methyltransferase	Comt	4.6	1.2
37	rc_AI230614_s_at/// rc_AI112173_at	NM_001002829	RAS-like family 11 member A	Rasl11a	3.0	1.1
38	rc_AA799511_g_at	–	–	–	1.8	2.6
39	rc_AA893871_at	–	–	–	1.4	2.5

Table 2 Aorta-dominant genes

Probe set ID	RefSeq transcript ID	Gene title	Gene symbol	Fold difference between aorta and DA		
				e19	e21	
1	X15939_r_at//rc_AI104924_f_at//rc_AI103920_f_at//rc_AA891522_f_at	NM_017239	Myosin heavy chain, polypeptide 6, cardiac muscle, alpha	Myh6	2.2	23.8
2	X80130cnds_i_at//rc_AI104567_g_at//rc_AA866452_s_at	XM_215801	Actin alpha cardiac 1	Actc1	3.0	18.4
3	M93638_at	NM_183333	Keratin complex 2, basic, gene 5	Krt2-5	6.9	16.8
4	rc_AA891242_g_at//rc_AA891242_at	NM_001106017	Myosin, light polypeptide 7, regulatory	My17	3.2	15.3
5	X15939_f_at	NM_017240	Myosin, heavy polypeptide 7, cardiac muscle, beta	Myh7	1.9	13.2
6	D78159mRNA_s_at	NM_001008806	Type II keratin Kb4	Kb4	1.5	9.1
7	X15512_at	NM_012824	Apolipoprotein C-I	Apoc1	0.5	7.4
8	U67914_at	XM_342219	Carboxypeptidase A3	Cpa3	1.7	7.2
9	rc_AI169372_g_at	NM_031839	Cytochrome P450, family 2, subfamily c, polypeptide 23	Cyp2c23	0.5	5.5
10	K01933_at	NM_012582	Haptoglobin	Hp	0.5	5.1
11	M80829_at	NM_012676	Troponin T2, cardiac	Tnn2	1.3	4.8
12	X00975_g_at//X07314cnds_at	NM_001035252	Myosin, light polypeptide 2	My12	5.1	4.8
13	M24852_at	NM_013002	Purkinje cell protein 4	Pcp4	4.7	4.8
14	M92074_g_at	NM_017144	Troponin I, cardiac	Tnni3	2.1	4.6
15	rc_AA945054_s_at	NM_022245	Cytochrome b-5	Cyb5	0.8	4.1
16	S76779_s_at	NM_138828	Apolipoprotein E	Apoe	1.3	3.9
17	AF014503_at	NM_053611	Nuclear protein 1	Nupr1	3.2	3.9
18	X02412_at	NM_019131	Tropomyosin 1, alpha	Tpm1	1.0	3.7
19	D00752_at	NM_182474	Serine protease inhibitor	Spin2a	0.5	3.6
20	D89730_at	NM_001012039	Epidermal growth factor-containing fibulin-like extracellular matrix protein 1 (predicted)	Efemp1_predicted	4.0	3.6
21	M14656_at	NM_012881	Secreted phosphoprotein 1	Spp1	4.7	3.4
22	X81448cnds_at//rc_AI072634_at	NM_053976	Keratin complex 1, acidic, gene 18	Krt1-18	1.5	3.1
23	rc_AA946368_at	XM_575338	Similar to fatty acid translocase/CD36	LOC499984	1.9	3.1
24	M91595exon_s_at//J04486_at//A09811cnds_s_at	NM_013122	Insulin-like growth factor binding protein 2	Igfbp2	2.1	3.1
25	U30938_at	NM_013066	Microtubule-associated protein 2	Mtap2	2.1	3.0
26	Y12502cnds_at	NM_021698	Coagulation factor XIII, A1 subunit	F13a	2.8	2.9
27	M84719_at	NM_012792	Flavin containing monooxygenase 1	Fmo1	4.4	2.8
28	M91652complete_seq_at	NM_017073	Glutamine synthetase 1	Glul	1.8	2.8
29	J03752_at	NM_134349	Microsomal glutathione S-transferase 1	Mgst1	1.1	2.7
30	AF072411_g_at//rc_AA925752_at	NM_031561// XM_575338// XM_575339	CD36 antigen//similar to fatty acid translocase/CD36//similar to fatty acid translocase/CD36	Cd36//LOC499984// LOC499985	1.7	2.7
31	L19998_g_at//L19998_at	NM_031834	Sulfotransferase family 1A, phenol-preferring, member 1	Sult1a1	2.9	2.7
32	L25387_g_at	NM_206847	Phosphofructokinase, platelet	Pfkp	1.7	2.6
33	rc_AI230247_s_at	NM_019192	Selenoprotein P, plasma, 1	Sepp1	2.2	2.6
34	rc_AI237731_s_at//L03294_g_at//L03294_at	NM_012598	Lipoprotein lipase	Lpl	0.9	2.6
35	M83680_at	NM_053589	RAB14, member RAS oncogene family	Rab14	2.6	2.6
36	rc_AI639532_at	XM_215935	Troponin C2, fast (predicted)	Tnnc2_predicted	4.7	2.5
37	X71127_g_at	NM_019262	Complement component 1, q subcomponent, beta polypeptide	C1qb	3.2	2.5
38	AB000113_at	NM_017217	Solute carrier family 7 (cationic amino acid transporter, y + system), member 3	Slc7a3	1.6	2.5
39	rc_AA894092_at	XM_342245	Periostin, osteoblast specific factor (predicted)	Postn_predicted	2.5	2.5
40	M32062_g_at	NM_053843// XM_573502// XM_573503	Fc receptor, IgG, low affinity III//Fc gamma receptor II beta//similar to low affinity immunoglobulin gamma Fc region receptor III precursor (IgG Fc receptor III) (Fc-gamma RIII) (FcRIII)	Fcgr3// LOC498276// LOC498277	3.2	2.4
41	M24353_g_at	XM_343636	Mannosidase 2, alpha 1	Man2a1	4.7	2.3

Table 2 continued

Probe set ID	RefSeq transcript ID	Gene title	Gene symbol	Fold difference between aorta and DA	
				e19	e21
42 U77931_at	NM_147136	rRNA promoter binding protein	RGD:727924	3.0	2.3
43 M12098_s_at	NM_012604	Myosin, heavy polypeptide 3, skeletal muscle, embryonic	Myh3	2.7	2.1
44 AJ005396_at	XM_342325	Procollagen, type XI, alpha 1	Col11a1	3.1	2.1
45 rc_AA893230_at	XM_236325	Ceroid-lipofuscinosis, neuronal 6 (predicted)	Cln6_predicted	2.8	2.1
46 rc_AA875172_at	NM_053360	SH3-domain kinase binding protein 1	Sh3kbp1	2.6	2.1
47 AB012235_at//AB012234_g_at//AB012234_at	XM_213849	Nuclear factor I/X	Nfix	4.9	1.9
48 X00722_at	XM_578812	Similar to testin	LOC503278	2.8	1.9
49 M32062_at	NM_053843//XM_573502	Fc receptor, IgG, low affinity III//Fc gamma receptor II beta	Fcgr3//LOC498276	2.8	1.8
50 rc_AI013472_at//rc_AA924925_at	NM_138905	ER transmembrane protein Dri 42	Ppap2b	2.6	1.8
51 U62897_at	NM_012836	Carboxypeptidase D	Cpd	3.0	1.8
52 Z12298cds_s_at//X59859_r_at//X59859_i_at	NM_024129	Decorin	Dcn	2.7	1.7
53 M15797_at	XM_213954	Nidogen (entactin)	Nid	13.4	1.7
54 AF041066_at	NM_020082	Ribonuclease, RNase A family 4	Rnase4	2.9	1.7
55 U50842_at	XM_343427	Neural precursor cell expressed, developmentally downregulated gene 4A	Nedd4a	3.9	1.6
56 rc_AA866443_at	NM_001008560	Protease, serine, 35 (predicted)	Prss35	4.2	1.6
57 S66184_s_at//rc_AI234060_s_at//rc_AI102814_at//rc_AA875582_at	NM_017061	Lysyl oxidase	Lox	3.2	1.6
58 rc_AI639314_at	XM_238213	Delangin (predicted)	NIPBL_predicted	2.5	1.6
59 rc_AA800908_at	XM_344450	Potassium channel tetramerisation domain containing 12 (predicted)	Kctd12_predicted	3.6	1.6
60 rc_AI029920_s_at	NM_012817	Insulin-like growth factor binding protein 5	Igfbp5	4.0	1.5
61 L10326_at	NM_019132	GNAS complex locus	Gnas	2.7	1.5
62 E00988mRNA_s_at	NM_031511	Insulin-like growth factor 2	Igf2	3.0	1.4
63 U01908cds_s_at//D43778exon#3_s_at//D16840_s_at	NM_012494	Angiotensin II receptor, type 2	Agtr2	4.4	1.4
64 rc_AI176461_s_at	NM_017211	Golgi apparatus protein 1	Glg1	3.3	1.4
65 U23146cds_s_at	NM_057103	A kinase (PRKA) anchor protein (gravin) 12	Akap12	2.9	1.4
66 rc_AA900750_s_at	NM_012760	Pleiomorphic adenoma gene-like 1	Plagl1	2.5	1.4
67 Z17223_at	NM_017149	Mesenchyme homeo box 2	Meox2	2.6	1.3
68 rc_AI171966_at	NM_198740	Major histocompatibility complex, class II, DM beta	RGD:735096	2.5	1.2
69 U35775_g_at//U35775_at	NM_031552	Adducin 3 (gamma)	Add3	5.3	1.2
70 U43534_at	NM_012894	Adenosine deaminase, RNA-specific, B1	Adarb1	2.7	0.8
71 AF004811_at	NM_030863	Moesin	Msn	2.9	0.7
72 X05831cds_at//U82612cds_at//M28259cds_at	NM_019143	Fibronectin 1	<td>3.9</td> <td>0.7</td>	3.9	0.7
73 U17604_at	NM_053865	Reticulon 1	Rtn1	2.6	0.7
74 X51531cds_g_at//X51531cds_at	-	-	-	4.4	30.2
75 rc_AA799865_at	-	Transcribed locus	-	1.3	2.7
76 X05472cds#2_at	-	-	-	7.2	2.4
77 rc_AA799406_at	XM_578859	Hypothetical protein LOC503325	LOC503325	2.6	1.3
78 rc_AA859921_at	-	-	-	3.3	0.9

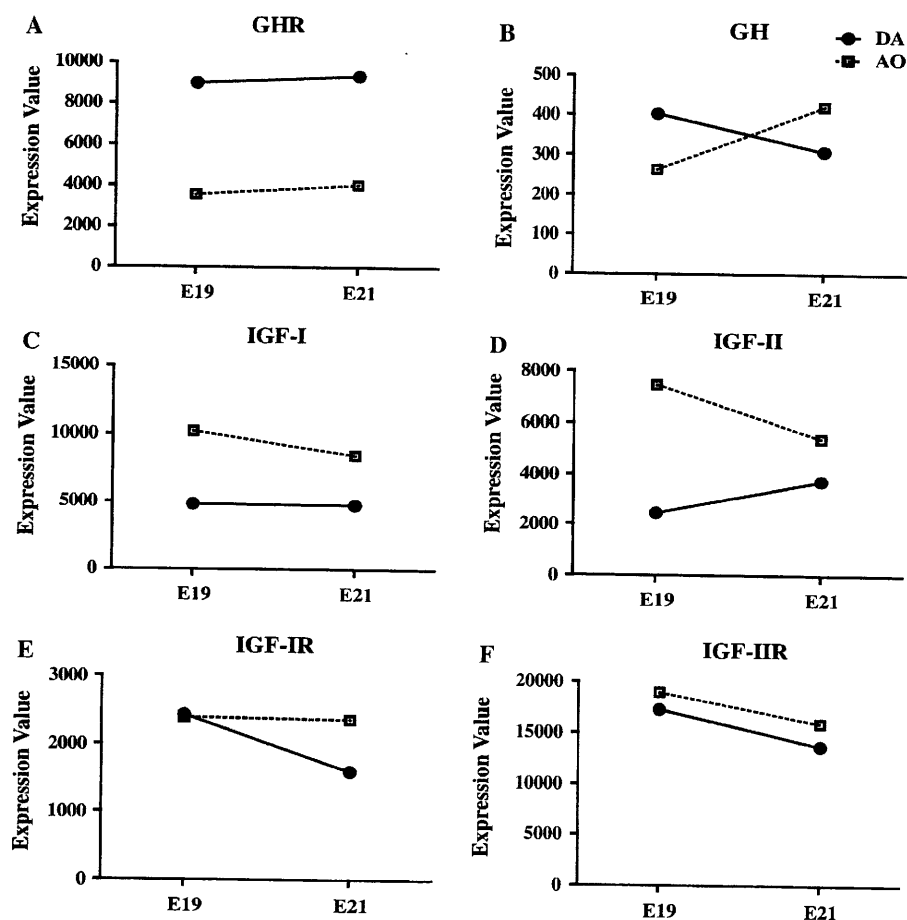
in the aorta at e19 and at e21 ($p < 0.05$ and $p < 0.001$, $n = 6-8$, respectively) (Fig. 2a).

We then examined the localization of GHR in the DA and the aorta at e19 and e21. GHR immunoreactivity was detected abundantly in the SMC layer and less in the endothelial cells of the DA (Fig. 2b).

GH promoted DA SMC migration, but not proliferation

SMC migration and proliferation play an essential role in intimal cushion formation of the DA, especially during late gestation. Therefore, we investigated the effects of GH on migration and proliferation using DA SMCs in primary

Fig. 1 The expression of growth hormone receptor and its related genes in the developing DA by DNA microarray analysis. **a** GHR (growth hormone receptor), **b** GH (growth hormone), **c** IGF (insulin-like growth factor)-I, **d** IGF-II, **e** IGF-IR (type I receptor), and **f** IGF-IIR (type II receptor) mRNA. *E19* Embryonic day 19, *E21* embryonic day 21, *DA* ductus arteriosus, *AO* aorta



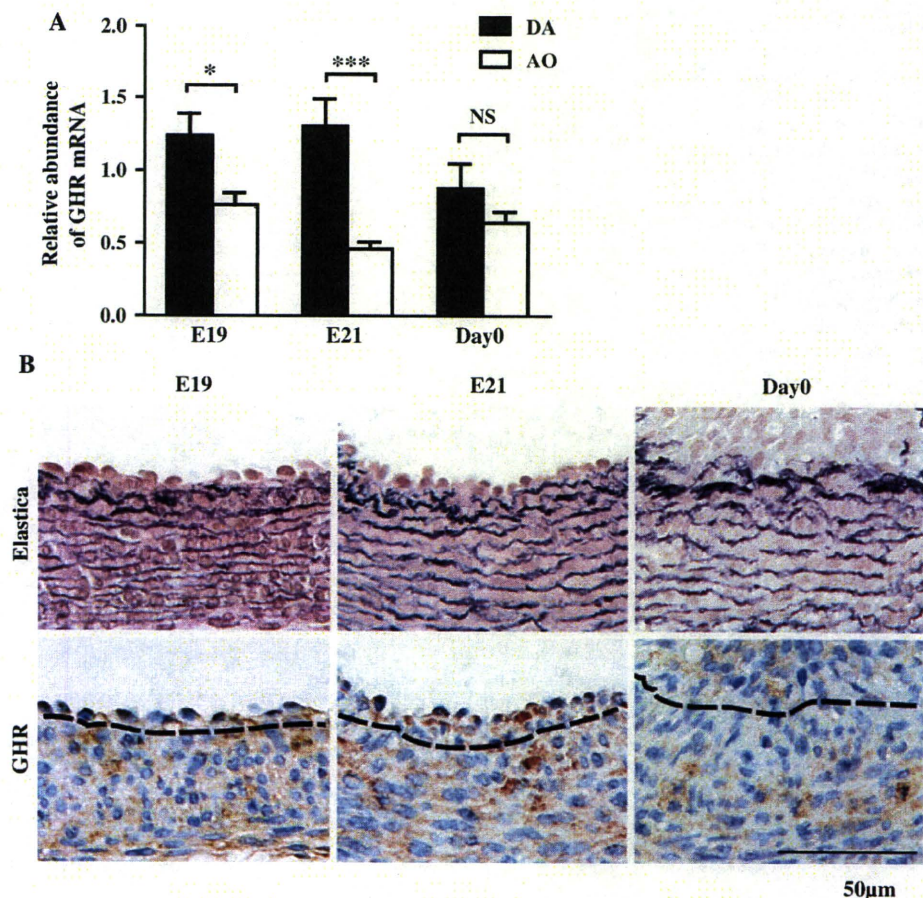
culture from rat DA at e21. We found that recombinant rat GH promoted migration of DA SMCs in a dose-dependent manner (Fig. 3a). However, the same amount of GH did not promote migration of aortic SMCs (Supplemental data 3). When DA SMCs were treated with platelet-derived growth factor BB (PDGF-BB) (10 ng/ml), a potent stimulator for SMC migration, SMC migration was significantly increased by 141% in DA SMCs. In contrast, [3 H]thymidine incorporation was unchanged in DA SMCs in the presence of recombinant rat GH (up to 200 ng/ml) (Fig. 3b). When DA SMCs were treated with 10% FBS, a potent stimulator for SMC proliferation, [3 H]thymidine incorporation was significantly increased by 112% in DA SMCs. Hyaluronan is an important component of the intimal cushion, and hyaluronan-rich matrices are essential for cell migration and proliferation in the DA [2]. Because our recent study revealed that PGE₁ is a potent stimulator for hyaluronan production in DA SMCs [2], we investigated whether or not GH altered hyaluronan production in DA SMCs. We found that GH had no effect on hyaluronan production in DA SMCs (data not shown).

Effect of GH on tissue-specific cytoskeletal genes in the DA and the aorta

Our DNA array analyses revealed that the expression of cytoskeletal genes was markedly different between the DA and the aorta. Because GH is known to regulate cytoskeletal organization [7], we examined whether or not GH affected such a tissue-specific expression of cytoskeletal genes in DA SMCs. We found that GH decreased the expression of DA-dominant cytoskeletal genes such as desmin, Fhl2, Actg2, and Myh11 in DA SMCs (Fig. 4a–d). Among the aorta-dominant sarcomere genes, we also found that GH decreased the expression of Myl2 and Tnnt2 mRNAs, increased the expression of Tnni3 mRNA, and exhibited no change in Myh7, Actc1, and Tnnc2 mRNAs (Fig. 5).

Because GH is known to inhibit the expression of skeletal muscle-specific proteins in a dose-dependent manner in satellite cells [8], we examined the effect of GH on the expression of smooth muscle-specific genes. We found that GH significantly decreased the expression of SM1, SM2, SM22, and h-caldesmon mRNAs, whereas GH did not

Fig. 2 Expression of GHR mRNA and protein in rat DA.
a Quantitative RT-PCR analyses of GHR. The expression level of GHR mRNA was maximal at E19 and E21 in the DA ($n = 6-8$).
b Immunohistological analysis of GHR protein in the rat DA at E19, E21, and Day0. GHR was detected in a brown color. Dark blue stain was counterstained with Mayer's hematoxylin. Scale bars 50 μ m. * $p < 0.05$, *** $p < 0.001$. Data are expressed as means \pm SEM. E19 Embryonic day 19, E21 embryonic day 21, Day0 at birth, DA ductus arteriosus, AO aorta, GHR growth hormone receptor, NS not significant



change the expression of SMemb mRNA (Fig. 6a–e). We also found that GH decreased the expression of myocardin mRNA (Fig. 6f), a transcriptional factor, which is sufficient for a smooth muscle-like contractile phenotype. To investigate whether the effect of GH on the expression of cytoskeletal genes is found in aortic SMCs, we also did the same experiment using SMCs from the rat aorta at e21. We also found a similar effect of GH on the expression of cytoskeletal genes in cultured rat aortic SMCs (Supplemental data 4, 5, and 6).

GH promoted intimal thickening of immature rat DA explants

To examine to what extent GH contributes to the intimal thickening of the DA, we administrated GH into the premature vessel explants containing the DA, the aorta, and the main pulmonary artery from fetuses at e19 (Fig. 7). We found that GH significantly promoted intimal thickening of the DA, but not the aorta when compared with the control (Fig. 7). It should be noted that the effect of GH on the intimal thickening was greater in the DA than in the aorta.

Discussion

Our microarray analyses uncovered gene expression profiles of the DA distinct from those of the aorta during fetal development. These gene expression profiles are considered to be the primary determinant of the different functional and morphological characteristics of the DA from the adjacent arteries. In fact, several unexpected genes that are known to be involved in tissue differentiation were identified as having a DA-dominant expression pattern. It is of note that among them GHR exhibited the highest difference in expression between the rat developing DA and the adjacent aorta. Although the expression levels of GH mRNA were slightly higher in the aorta than in the DA at e21, the difference did not reach statistical significance. We think that total GH-GHR signals are higher in the DA than in the aorta due to the predominant expression of GHR in the DA during gestation. Accordingly, we hypothesized that GH stimulation via GHR may be involved in DA remodeling and that it may play a role in the specification of the DA from other arteries. During gestation, the serum GH concentration of fetuses increased gradually as

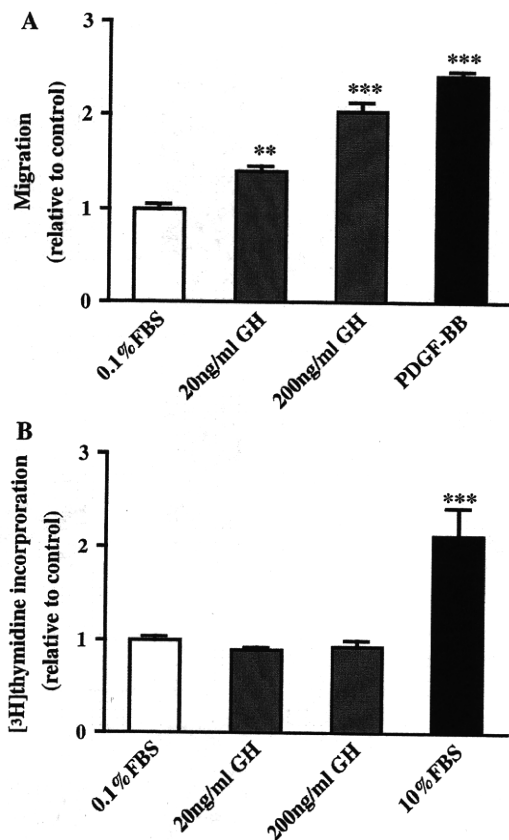


Fig. 3 GH promoted DA SMC migration, but not proliferation. **a** Effects of growth hormone (GH) stimulation on DA SMC migration. DA SMC migration was significantly increased in the presence of GH at a concentration of 20 or 200 ng/ml ($n = 6$). PDGF-BB at a concentration of 10 ng/ml was used as a positive control. **b** The effect of GH on proliferation of rat DA SMCs. GH did not promote cell proliferation in DA SMCs ($n = 6-8$); 10% FBS was used as a positive control. ** $p < 0.01$, *** $p < 0.001$ versus 0.1% FBS. Data are expressed as means \pm SEM. FBS Fetal bovine serum

pregnancy advanced and declined before parturition in many species [9–11]. In rodents, the fetal pituitary gland starts to secrete GH from e15, whereas many tissues, including vascular endothelial and SMCs, locally produce GH beginning in relatively early embryogenesis [12]. Even though the level of serum GH in a fetus is almost comparable to or slightly less than that in an adolescent [9–11], GH has been regarded as of little functional significance in fetal growth [13]. A growing body of evidence, however, has revealed the importance of GH in tissue differentiation during fetal development. First, many fetal tissues, including vascular endothelium and smooth muscle, express GHR [14, 15]. Therefore, GH likely activates an intracellular signal pathway through GHR in fetuses. Second, a considerable number of studies have demonstrated that fetal tissues indeed respond to GH in vitro [12, 16], which was observed in the present study. Third,

GHR knockout mice exhibit functional and morphological changes in both heart and vasculature [17]. These results led us to explore the role of GH in DA development, especially in its vascular remodeling.

Numerous previous studies have demonstrated that GH plays a role in angiogenesis [18] and that GH deficiency or excess increases the risk of cardiovascular morbidity and mortality [19, 20]. However, the role of GH in the vascular remodeling of the developmental arteries has not yet been precisely evaluated. The present study revealed that GH promoted the migration of DA SMCs and then intimal cushion formation in DA explants. In terms of the effect of GH on SMC migration, data from previous studies are very limited [21–23]. Although the precise mechanism still has not been investigated, it can be assumed that the different responses to GH in various cell types are dependent on the GHR expression levels.

Intimal thickness is a hallmark of physiological vascular remodeling of the DA during late gestation [2]. Although the present study demonstrated that GH promotes intimal cushion formation of the ex vivo DA explants, previous clinical studies have shown that the effect of GH on pathological intimal thickness is equivocal. Increases in the carotid intimal media thickness were observed in patients with acromegaly [24]. In contrast, patients with either childhood- or adulthood-onset GH deficiency also exhibited increased intima-media thickness and endothelial dysfunction [19, 25]. Therefore, adequate levels of GH could be important to maintain normal morphology of mature arteries. The present study indicates that GH in the culture media at a concentration of 200 ng/ml is sufficient to promote the physiological intimal cushion formation of the DA through increasing SMC migration.

It is known that the differentiation of DA SMCs precedes that of other arteries [26, 27]. Nevertheless, we were surprised by the considerable number of cardiac-type sarcomere genes expressed in the fetal DA, although it was much less than that in the aorta. The present data suggest that prior to complete differentiation, vascular SMCs may retain a high degree of plasticity, which allows them to modulate their phenotype [28]. Interestingly, through the in vitro experiment, we found that the effect of GH on the expression of the cytoskeletal genes was not always consistent with the tissue-dominant expression patterns that were identified by DNA microarray analysis. Therefore, factor(s) other than GH may determine the tissue-specific expression pattern of cytoskeletal genes in the DA and the aorta.

More importantly, we found that GH downregulated the genes involved in a smooth muscle-like contractile phenotype. Consistent with the result, we also found that GH downregulated myocardin mRNA, which is sufficient for a smooth muscle-like contractile phenotype [28]. In addition

Fig. 4 Effect of GH on the cytoskeletal genes and smooth muscle-specific genes in DA SMCs. **a** Desmin, **b** Fhl2, **c** Actg2, **d** Myh11. ($n = 15$). $*p < 0.05$, $***p < 0.001$. Data are expressed as means \pm SEM. *GH* Growth hormone, *CTRL* control, *DA SMC* ductus arteriosus smooth muscle cell, *NS* not significant

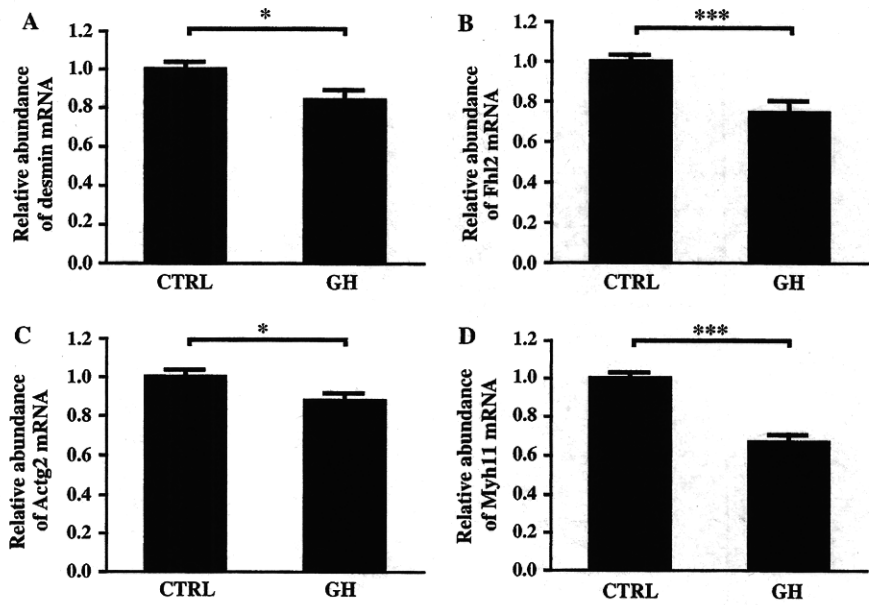


Fig. 5 Effect of GH on the expression of the aorta-dominant sarcomere genes in DA SMCs. **a** Myl2, **b** Tnnt2, **c** Tnni3, **d** Myh7, **e** Actc1, and **f** Tnnc2. ($n = 15$). $**p < 0.01$, $***p < 0.001$. Data are expressed as means \pm SEM. *GH* Growth hormone, *CTRL* control, *DA SMC* ductus arteriosus smooth muscle cell, *NS* not significant

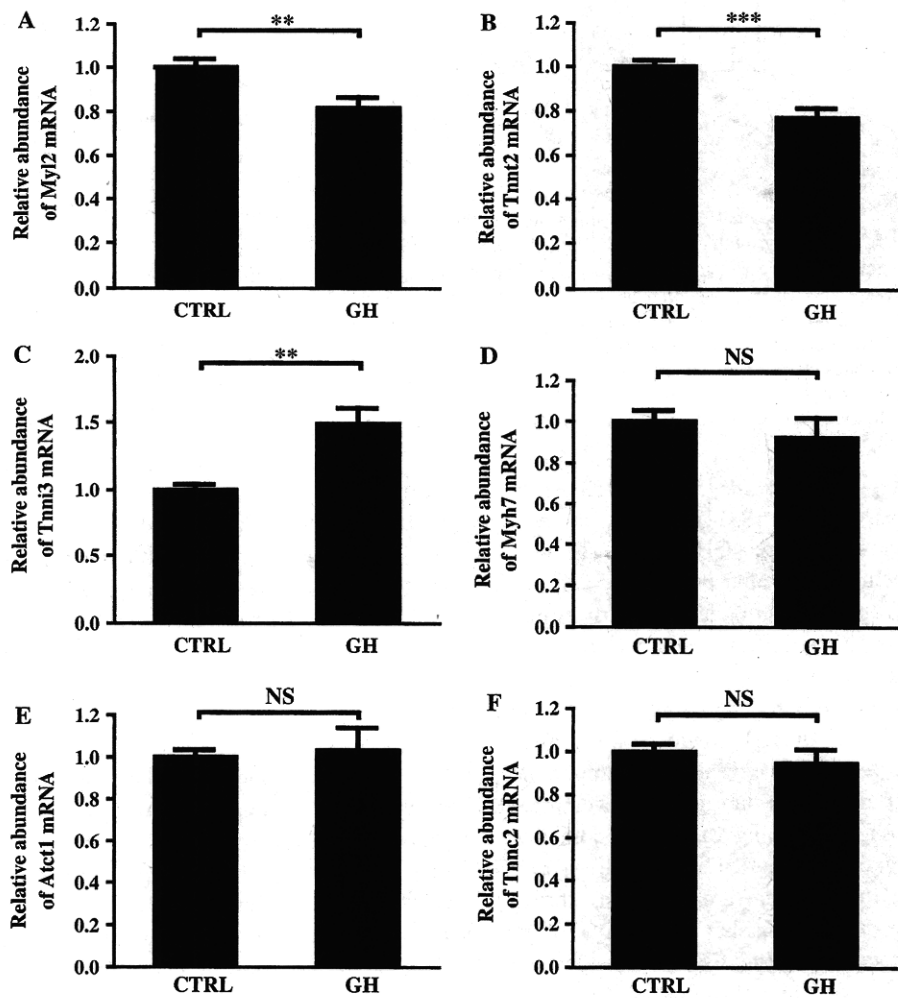
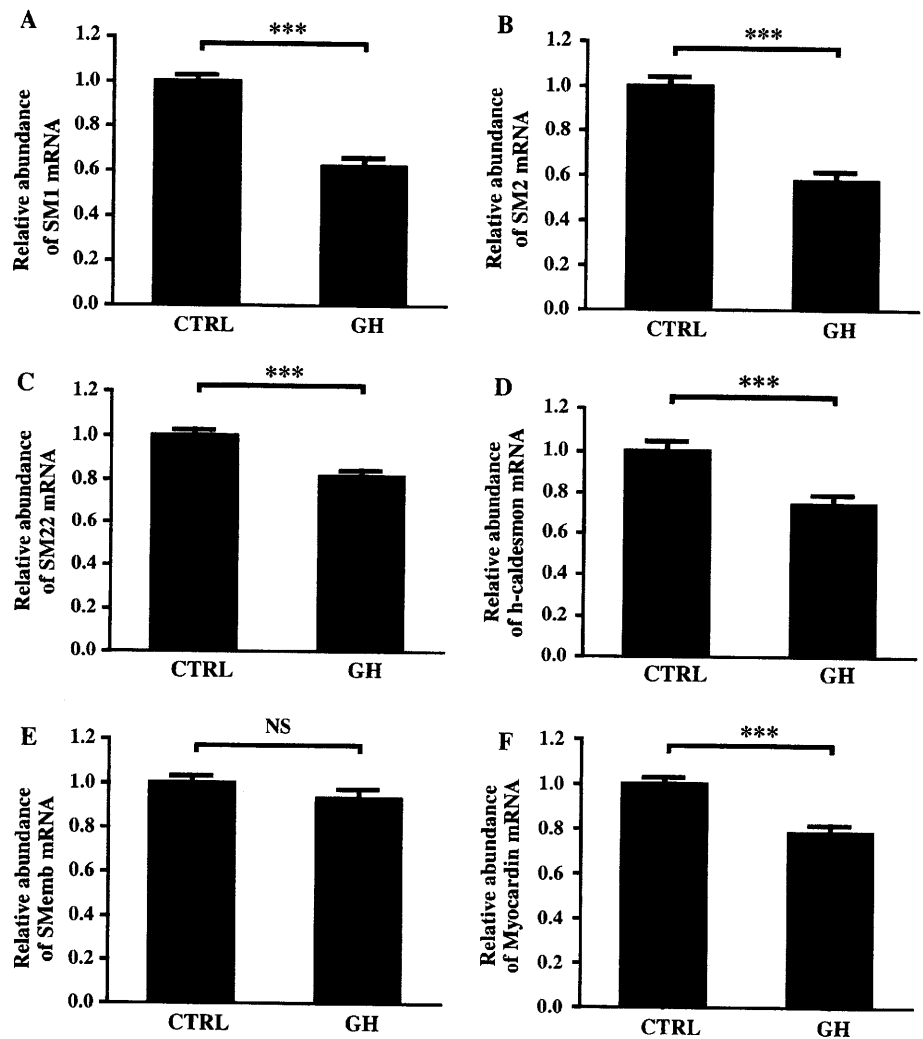


Fig. 6 Effect of GH on the cytoskeletal genes and smooth muscle-specific genes in DA SMCs. **a** SM1, **b** SM2, **c** SM22, **d** h-caldesmon, **e** SMemb, **f** Myocardin. ($n = 15$). * $p < 0.05$, *** $p < 0.001$. Data are expressed as means \pm SEM. GH Growth hormone, CTRL control, DA SMC ductus arteriosus smooth muscle cell, NS not significant

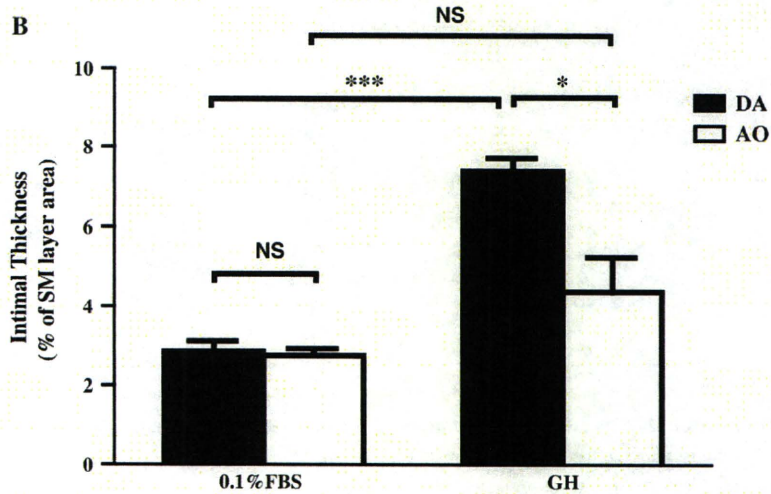
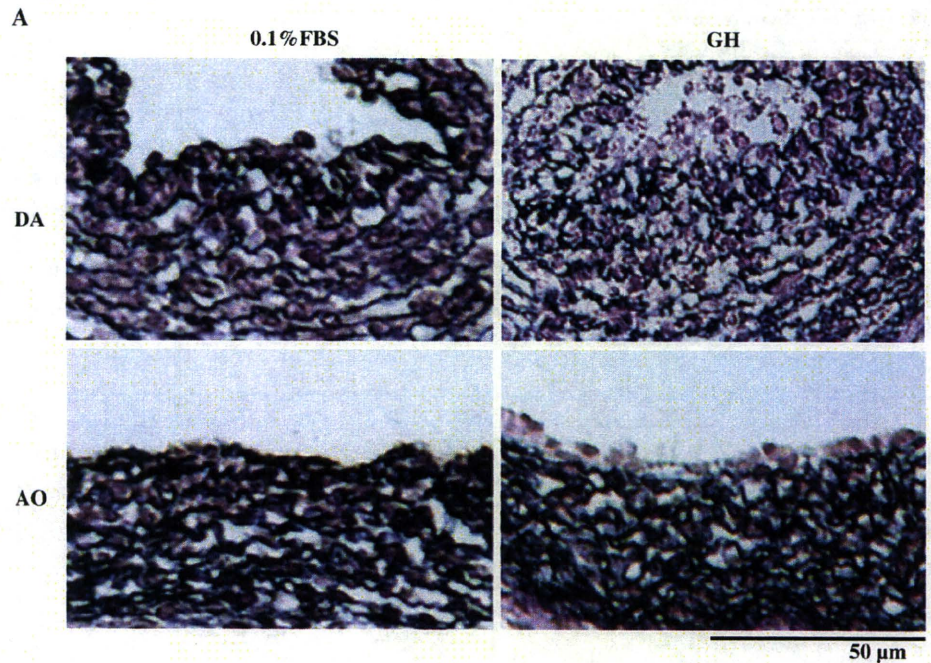


to this observation, Halevy et al. [8] demonstrated that GH inhibited the gene expression of myogenin and the expression of skeletal muscle-specific proteins in a dose-dependent manner in satellite cells. These data suggested that during muscle differentiation, GH inhibited the muscle-specific differentiation at its final stage to retain its synthetic phenotype. We propose that DA SMCs consist of distinct types that depend on their localization. During the progression of intimal cushion formation, DA SMCs in the inner layer are a synthetic phenotype that is highly proliferative and can migrate easily. The GH-GHR signal helps these cells remain a synthetic phenotype. Further studies are required to prove this idea.

Although the present microarray analyses uncovered distinct gene expression profiles of the DA from those of the aorta, several gene profiles are different from a previous report demonstrating the transcriptional profiles between the rat DA and the aorta of premature fetuses

and neonates using the same DNA microarray plates we used [3]. For example, Costa et al. demonstrated that Myl2 and Myh7 are predominantly expressed in the rat aorta at e19, but our data showed the opposite result. In addition, the tissue-specific genes that we identified overlap very little with their findings. We do not have a reasonable explanation for this discrepancy. It should be noted that the present study identified several expected genes that are known to be predominantly expressed in the DA, such as prostaglandin E receptor 4, endothelin-1, and Kcnj8 (potassium inwardly rectifying channel, sub-family J, member 8). In contrast, Costa et al. did not identify this expected DA-dominant gene in the data from their microarray analysis. Furthermore, we also confirmed by quantitative RT-PCR analysis that, using different sets of RNA samples, the expression of several DA-dominant genes was higher in the DA than in the aorta. Therefore, we are confident that we provided

Fig. 7 Effects of GH-mediated intimal thickening of immature rat DA and aorta explants. **a** Elastica staining of the DA and the aorta. GH at a concentration of 200 ng/ml. Scale bars 50 μ m. **b** GH significantly promoted intimal thickening of the DA, but not the aorta. ($n = 4-6$). * $p < 0.05$, *** $p < 0.001$. Data are expressed as means \pm SEM. DA Ductus arteriosus, AO aorta, GH growth hormone, NS not significant



reliable data regarding the transcriptional profiles of the developing rat DA.

In conclusion, our study highlighted the distinct transcriptional profiles of the DA. In addition to the expected genes, microarray analysis revealed many genes whose roles were previously unrecognized in the DA. Among them, we found that the GH-GHR signal plays a role in vascular remodeling of the DA by promoting migration of SMCs and the subsequent formation of intimal thickness and regulating the expression of cytoskeletal genes. Although further studies are needed to identify the role of other genes in the DA, our data provide a basis for understanding the molecular mechanisms underlying the

differentiation and remodeling of the DA and for inventing the novel targets that regulate the contraction of the DA in affected children.

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EUにおける細胞・組織加工製品の規制動向

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Regulation of Cell/Tissue-Based Medicinal Products in the European Union

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はじめに

バイオテクノロジーや幹細胞学等の進展に伴い、再生医療・細胞治療などの先端医療で使用することを目的として、培養・活性化等の加工が施された生細胞を含む医薬品・医療機器(細胞・組織加工製品)が国内外で数多く開発されつつあり、今まで治療が困難であった疾患や重度の損傷への高い効果が期待されている。これらの開発の勢いに呼応し、細胞・組織加工製品の品質及び安全性を確保するための行政施策・規制をいち早く整備することは、細胞・組織加工製品の实用化を促進して患者のもとにいち早く届けるという意味の上からも、製品の国際競争力確保の意味の上からも大きな課題である。また、製品の効率的な国際流通を視野に入れた場合、世界各国・各地域における承認審査での有効性・安全性・品質評価に関する考え方についての理解及び国際的協調が不可欠である。

欧州連合(EU)では、細胞・組織加工製品は体細胞治療薬(somatic cellular therapy products)又は組織工学製品(tissue engineered products)の範疇に分類されている。従来、体細胞治療薬は遺伝子治療薬とともに先端医療医薬品(ATMP, advanced therapy medicinal products)という医薬品の一類型に分類されていたが、2008年12月より組織工学製品もATMPとして規制を受けることになった。また、同時にATMPの審査に特化し

た先端医療委員会(CAT)が創設されるなど、積極的な開発支援策が取られている。本稿ではEUにおける、これらの新しい取り組みについて概説する。

1. ATMPの規制の枠組み

EUでは、ATMPは欧州医薬品庁(EMA, European Medicines Agency)が販売承認審査を担当する。2008年12月以前はATMPの範疇に含まれる製品は、遺伝子治療薬(gene therapy products)と体細胞治療薬のみで組織工学製品が含まれておらず^{1,2)}、また、これらの製品の販売承認審査における評価基準に関して、EU加盟国間で統一がとれていなかった点が問題とされてきた。なおEUでは、医療機器に関しては、いずれかの加盟国より認定された民間の第三者認証機関の認証を受ければEU内の国境を越えた流通が可能となっており、国による審査は行われていないが、組織工学製品については医薬品¹⁾に分類されるか、医療機器^{3,4)}に分類されるか、その判断は加盟国によりまちまちであった。

欧州委員会(EC)はこれらの問題を、EU内で国境を越えた製品の流通を展開する際の大きな障壁であると考え、その解決策として2007年、ATMPの販売承認規制を定めるRegulation(EC)No1394/2007³⁾を定めた。Regulation(EC)No1394/2007は、組織工学製品をATMPの範疇に加えること、及びATMPについては加盟国にお

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