4）二次治療以降

アントラサイクリンあるいはタキサンに対し不応性となった場合，ビノレルビン，カペシタビン，テガフール・ギメラシル・オテラシルカリウム配合カプセル剤，イリノテカン，マイトマイシンCなどが考慮される。あるいは，ゲムシタビンも乳癌での適応は未承認であるが，有効な薬剤である。奏効率は10～30%，奏効期間は6ヵ月以内である。いずれの薬剤を優先させるべきか，明確なデータは得られていない。副作用や患者の全身状態などを考慮したうえで薬剤を選択すべきである。

5）経口フルオロウラシル系薬剤

経口フッ化ピリミジン系抗癌剤に分類されるものには，フルオロウラシル，テガフール・ウラシル配合剤，ドキシフルリジン，カペシタビン，テガフール・ギメラシル・オテラシルカリウム配合カプセル剤がある。カペシタビンはアントラサイクリン，タキサン耐性乳癌に対する治療薬として米国食品医薬品局(FDA)で承認されている唯一の経口フッ化ピリミジン系抗癌剤で，国内でも2003年に承認されている。本剤は，未変化体として腸管から吸収され，肝に局在するカルボキシエステラーゼにより5'-DFCRに変換され，肝あるいは腫瘍組織でシチジンデアミナーゼによりドキシフルリジンに変換される。ドキシフルリジンは腫瘍組織で高い酵素活性を示すチミジンホスホリラーゼによりフルオロウラシルに変換され作用する。これにより，腸管毒性・骨髄毒性が軽減され，腫瘍組織で効果を発揮する。アントラサイクリン耐性乳癌を対象として，ドセタキセルとカペシタビンの併用とドセタキセル単剤との比較検討を行った第3相試験が行われている。それによると，無増悪期間(6.1ヵ月 vs 4.2ヵ月，P＝0.0001)，全生存期間(14.5ヵ月 vs 11.5ヵ月，P＝0.126)ともに併用群が上回っていた。わが国では，進行再発乳癌を対象とした後期第2相試験やドセタキセル耐性乳癌に対する第2相試験が行われているが，奏効率はそれぞれ28.3%，20.0%であり，無増悪期間は155日，84日であった。経口投与という簡便性と比較的少ない毒性から，転移・再発乳癌に対する治療として有用な薬剤である。現在，他の抗癌剤との併用や術後補助療法あるいは術前療法などのセッティングでさまざまな検討が計画，実施されている。

6）ビノレルビン

ビノレルビンはビンアルカロイド系抗癌剤に分類され，アントラサイクリン，タキサン耐性の進行再発乳癌に対し，わが国でも承認されている。他のビンアルカロイド系抗癌剤と同様，微小管の形成を阻害(脱重合)することにより抗腫瘍効果を発揮する。25mg/m²をday 1，8に投与する3週間ごとのレジメンが用いられる。有害事象としては，静脈炎，骨髄抑制などがあるが，他のビンアルカロイド系抗癌剤にみられるような神経毒性などは軽減されている。単剤で用いた場合の奏効率は，ファーストラインでは35～59%，セカンドライン以降では16～40%と報告されている。アントラサイクリン，タキサン，あるいはゲムシタビンなどの他の抗癌剤との併用や，トラスツズマブとの併用などが試みられており，良好な成績が得られてきている。

7）分子標的治療

乳癌は分子標的治療薬としてトラスツズマブがいち早く導入され，現在では地位を確固たるものとしている。現在，臨床試験にてさらなる分子標的治療薬が検証されており，その効果が期待されている。

トラスツズマブは，その相乗効果により，特に化学療法との併用で高い治療効果が得られている。しかし，単剤でも毒性が比較的少なく高い治療効果を得ることが可能である。化学療法からトラスツズマブへの逐次投与，化学療法とトラスツズマブの同時投与に関してはその有効性が示されているが，トラスツズマブから化学療法への逐次投与についてはこれまで報告がなかった。最近，HERTAX試験で，ドセタキセル＋トラスツズマブを治療開始時から投与する同時併用群とトラスツズマブ単剤で開始し，抵抗性となった後，ドセタキセル単剤に変更する逐次併用群との比較が行われた。奏効率は，同時併用群73%，逐次併用群50%と同時併用群で高かった(P＝0.02)。しかし，無増悪生存期間は，同時併用群9.4ヵ月，逐次併用群はドセタキセルまで含めると10.8ヵ月とほぼ同様の成績を示し，全生存期間も有意差を認めなかった(30.5ヵ月 vs 20.2ヵ月)。さらに有害事象については，神経毒性が同時併用群で8%にみられたが，逐次併用群ではみられなかった。トラスツズマブ単剤で病勢進行となってから化学療法へスイッチする方法の有効性と安全性を探索することを目的とした無作為化第2相試験

ではあるが，転移・再発乳癌の治療目的がQOLを維持しながらの延命にあるとすれば，トラスツズマブ単剤で治療を開始することは，ある群の患者に対しては有用な選択肢になりうることを示唆しており，今後のさらなる検討が待たれる。

ベバシズマブはVEGF(vascular endothelial growth factor)をターゲットとした薬剤であり，大腸癌にてその効果が確認されている。乳癌については，E2100試験で再発・転移乳癌を対象とした一次治療としてパクリタキセル±ベバシズマブの比較検討が行われており，奏効率(36.9% vs 21.2%)，無増悪生存期間(11.8 vs 5.9ヵ月，ハザード比0.60，P<0.001)と，ベバシズマブ併用による有効性が示されている。AVADO試験では，同様に一次治療としてドセタキセル±ベバシズマブの比較が行われている。E2100試験とはコントロールをプラセボとしている点，二次治療としてベバシズマブ投与を可能としている点が異なる。追跡期間が中央値10.2ヵ月とまだ短いが，プラセボ群・ベバシズマブ7.5mg/kg併用群・ベバシズマブ15mg/kg併用群について，奏効率(44 vs 55 vs 63%)，無増悪生存期間(8.0 vs 8.7 vs 8.8ヵ月)と，いずれも有意にベバシズマブ投与群で良好な成績が得られている。

LapatinibはHER1とHER2の両受容体に対して阻害作用をもつ薬剤であり，HER2遺伝子増幅のみられる局所進行性または転移性乳癌にて臨床効果が期待できることが報告されている。Lapatinibを第1選択薬として投与した試験での中間解析にて，40%の奏効率を認めている。

また，VEGF，PDGF(platelet-derived growth factor)，Kit，Flt3(FMS-like tyrosine kinase-3)などのキナーゼを阻害するスニチニブは，アントラサイクリンやタキサン抵抗性乳癌に対して14%の奏効率が報告されている。その他にも多数の薬剤が現在検討中であり，その結果が待たれる。

1．周術期治療

術前化学療法によりpCRの得られた症例は予後良好であるが，pCRの得られなかった，いわゆるnon-pCR症例をどのように扱うかという問題がある。特に，ER陽性，HER2陰性の症例は，FEC→ドセタキセルなどのアントラサイクリンとタキサンを含むレジメンによるpCR率が比較的低い。こうした症例で，治療後に腫瘍の残存を認め，さらに腋窩リンパ節転移の残存を認める場合などには，術後療法をどうするかということについて判断が難しい。術前化学療法に対する早期の反応性が芳しくない場合には，その後の治療を再検討しpCR率そのものを向上させる工夫が必要になると思われる。また，pCRが得られなかった際に，術後療法として化学療法を追加するべきか否か，追加する場合にはどのようなレジメンがよいのか，という点については，さらなる検討を待つ必要がある。

また，ER・PgR・HER2陰性のいわゆるtriple negativeの症例は，ホルモン療法，トラスツズマブなどのいわゆる標的療法の適応とならず治療選択肢に乏しいため，必然的に化学療法に対する期待が大きい。しかし，triple negative乳癌は，化学療法そのものに対する反応性は比較的良好であるにもかかわらず，予後は不良で比較的早期に転移・再発をきたす傾向にある。たとえば術前化学療法における検討では，triple negative症例はnon-triple negative症例に比較して，高率にpCRが得られている(22 vs 11%，P=0.034)。しかしながら，その後の再発・生存についてはtriple negativeで有意に不良であった。ただし，triple negativeであってもpCRの得られた症例についてはnon-triple negativeと予後に変わりがないことも示されている。Triple negativeの半数以上を占めるbasal-likeは特に予後不良であることが知られており，現時点ではこうした症例に対する特異的な治療法は確立されていない。Basal-likeはその背景にBRCA1機能不全が関与している可能性が指摘されており，プラチナ製剤などのDNA傷害性の薬剤による治療効果が期待され，タキサンとプラチナ製剤との比較検討が現在進行中である。また，セツキシマブ，ベバシズマブ，スニチニブ，Dasatinib(本邦未承認)などの分子標的治療薬についても現在検討が行われており，その結果が期待される。

個々の症例に最適な治療法を選択し，化学療法の成績向上を図るためには，さらなる治療効果予測因子の開発が必要であると思われる。たとえば，アントラサイクリ

ンに対するTOPO2Aあるいは，タキサンに対するmicrotubule-associated protein tauなどについて，その有用性が検討されてきている。また，薬剤の代謝・活性に着目し，代謝酵素活性や薬剤の血中濃度による調節など，宿主の因子からのアプローチなども今後重要性が高まってくるものと思われる。

また，ゾレドロン酸は骨吸収を抑制し，骨関連事象の発現を抑制する効果から骨転移に対する治療薬として承認されているが，抗腫瘍効果や転移抑制効果も有することが動物実験などから示されている。ABCSG-12試験は，閉経前ホルモン受容体陽性乳癌を対象として，卵巣機能抑制下でのタモキシフェンとアナストロゾールの効果を比較した試験であるが，同時にゾレドロン酸の上乗せ効果も検討している。タモキシフェンとアナストロゾールの比較については，ATAC試験とは異なり，閉経前患者では無病生存率に有意差を見出すことはできなかった。しかしながら，ゾレドロン酸を追加することにより，無病生存率については，そのリスクを36%低下させることが示されている(ハザード比0.643，95%信頼区間0.46-0.91，P=0.011)。

これまで，同じビスフォスフォネート製剤であるClodronate(本邦未承認)についても，予後への影響を検討した試験がいくつかみられた。Dielらは，早期乳癌を対象としてClodronateを補助療法で使用した場合，死亡あるいは転移のリスクを低下させることを報告しており，さらに骨以外の転移リスクも低下させることを示した。しかし，このClodronateの再発抑制効果については議論のあるところであり，同様の臨床試験の結果をまとめたメタアナリシスでは，死亡あるいは転移のリスクには影響を及ぼさないとしている。また，コクラン・レビューでも，ビスフォスフォネートは，骨関連事象の発生リスクを低下させるが，転移リスクを低下させないとしている。したがって，ゾレドロン酸の転移抑制効果についても，今後のさらなる検討が必要と思われるが，ABCSG-12試験の結果は1つの可能性を示唆するものとして注目に値する。

2．進行再発乳癌治療

脳転移に対しての化学療法の効果は，一般にはあまり期待できない。HER2陽性乳癌に対してトラスツズマブは非常に有用な薬剤であるが，術後補助療法での検討ではトラスツズマブ投与群は非投与群に比較してむしろ脳転移のリスクが高いとするものもある。したがって，全身の治療効果に相反して脳転移が出現する可能性もあり注意が必要である。現時点では，脳転移に対して特異的な薬物療法はなく，手術やラジオサージャリーなどの放射線治療が行われている。ハイリスク症例に対しては，予防的全脳照射の可能性が現在検討されており，preliminaryなデータではあるが，照射により脳転移の頻度が抑えられている。一方，Lapatinibは小分子であるため血液脳関門の通過が可能であり，脳転移に対する効果が期待されている。

進行再発乳癌の化学療法は，life-threateningな状況やホルモン療法不応性の場合に考慮されるが，life-threateningではないが病勢が進行している場合などには，化学療法の適応に関して判断が難しい。また，進行再発乳癌の化学療法は一般には病変増悪あるいは継続困難な有害事象が生じないかぎりは継続するのが基本であるが，長期にわたり完全寛解に近い奏効が得られている際などには，いつまで継続すべきか，という問題が生じることがある。このように，化学療法の適応や至適投与期間についてはまだ解決すべき問題が存在する。病勢あるいは予後を的確に反映するモニタリングシステムの構築が望まれる。

そうしたなかで，転移再発乳癌の予後予測因子として，circulating tumor cells(CTC)が注目されている。最近の報告では，治療開始前のCTCが無増悪期間，全生存期間を予測する独立した因子であると報告されている。また，治療効果のモニタリングとしての有用性も検討されてきており，その結果が期待されている。

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分子標的治療薬への期待

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●ラパチニブ
●ペバシズマブ

Molecular target therapy of breast cancer.
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はじめに

分子標的治療とは生体内の特定の分子をターゲットとした治療法である。トラスツズマブの成功に続いて，ラパチニブやペバシズマブが登場しさらに耐性，効果予測，投与方法などに関してもさまざまな議論がなされるようになってきている。本項では乳癌領域における分子標的治療の現状，展望を進行中の臨床試験などをあげながら考察する。

Ⅰ．トラスツズマブの現状と効果予測

トラスツズマブは，HER2(human epidermal growth factor receptor 2)の細胞外ドメインに対して作成されたマウス由来モノクローナル抗体(4D5)の抗原結合部位をヒト免疫グロブリン定常部に移植したヒト化抗体である。HER2陽性乳癌に対する補助療法としてのトラスツズマブの効果は欧米を中心に行われたB-31試験/N9831試験，BCIRG 006試験，FinHer試験や日本人も参加したHERA 試験といった大規模臨床試験において証明されており標準的な補助療法として確立している(表)[1-5]。投与期間や化学療法の併用/逐次投与に関してはこれらの試験結果が順次公表されるとともに明らかになっていくものと思われるが，これらの大規模試験にエントリーされなかった腫瘍径1 cm未満の症例や高齢者に対する有効性に関してはさらに今後の検討が必要である。

ASCO(American Society of Clinical Oncology)ガイドラインではトラスツズマブを使用する際には免疫組織化学染色法(IHC)もしくはfluorescent *in situ hybridization*(FISH)法を実施することが推奨されており，前述のHERA試験においてもIHC3＋/FISH未実施群，IHC2＋/FISH＋群，IHC3＋/FISH＋群すべての群において一貫してトラスツ

表．トラスツズマブを使用した術後補助療法試験の成績

試験名 レジメン	症例数 (人)	観察期間 (平均)	無再発期間 (ハザード比，p値)	全生存期間 (ハザード比，p値)
NSABP/N9831 AC→PH	3,351	2.9年	0.48 p<0.00001	0.65 p<0.0007
HERA Any CT→H	5,102	2年	0.64 p<0.00001	0.66 p<0.0115
BCIRG 006 AC→DH	3,222	2年	0.61 p<0.0001	0.59 p<0.004
BCIRG 006 DCaH			0.67 p<0.0003	0.66 p<0.0017
FinHer D or V+H→FEC	232	3年	0.42 p<0.01	0.41 p<0.07

A：ドキソルビシン，C：シクロフォスファミド，P：パクリタキセル，H：トラスツズマブ，D：ドセタキセル，Ca：カルボプラチン，CT：化学療法，V：ビノレルビン

ズマブの有効性が認められた。しかし，FISH比がトラスツズマブの3年間の無再発期間に及ぼす影響は有意ではなく，FISH比は効果予測因子とならない可能性も示唆されている[6]。また，骨髄微小転移や循環血中腫瘍細胞(Circulating Tumor Cell；CTC)と原発巣ではHER2過剰発現の状況が異なるという報告があり，原発巣においてHER2過剰発現を認めない症例においてもトラスツズマブが有効である可能性がある[7][8]。Disseminated Tumor Cell (DTC)およびCTCにおけるHER2過剰発現に効果予測因子としての可能性があることが示唆されている[9]。

Ⅱ．脳転移への対処

トラスツズマブは抗体薬であるため脳血液関門は通過しないとされている。

HERA試験，NSABP B-31/N9831試験の解析において初発転移巣としての脳転移は観察群30人(0.89%)に対して，トラスツズマブ投与群では54人(1.6%)であり，有意差は認めなかった[10]。しかし今後トラスツズマブが標準治療として施行されるようになると初発転移巣としての脳転移の割合が増加する可能性があり，脳転移のスクリーニング方法の標準化と同時に全身的治療としての脳転移に対する対処が必要になると思われる[11]。

HER1とHER2双方のチロシンキナーゼを阻害する薬剤として開発されたラパチニブはHER2陽性の局所進行および転移性乳癌に対する効果，特に脳転移に対する効果が期待されている。EGFRを過剰発現させた腫瘍細胞の脳転移マウスモデルに対してラパチニブを投与し，非投与群に比べ良好な効果が認められた[12]。

Ⅲ．ラパチニブによるトラスツズマブ耐性の克服の可能性

第三相試験のカペシタビンとの併用では，HER2陽性局所進行・転移性乳癌に対してカペシタビン(2,000mg/m^2 2週投与1週休薬を1サイクル)＋ラパチニブ(1,250mg/日)群とカペシタビン単剤(2,500mg/m^2 2週投与1週休薬を1サイクル)投与群で比較したところ，ラパチニブ併用群で有意に無進行期間の延長(4.3 vs. 6.2ヵ月)(HR＝0.57，p<0.001)を認めた。さらに初発転移巣としての脳転移はラパチニブ投与群において有意に減少したと報告されている(4 vs. 13人，p＝0.045)[13]。また，トラスツズマブ治療歴のある放射線治療抵抗性のHER2陽性脳転移症例241例に対してラパチニブ単剤投与(750mg/1日2回投与)を行ったところ19症例(7%)に50%以上の腫瘍縮小を認め，46症例において20%以上の腫瘍縮小を認めた。また7症例(18%)において16週間の無進行期間を認めている。さらに進行する症例においてはカペシタビンとの併用により良好な効果が示されている[14]。

パクリタキセルとの併用においても局所進行・転移性乳癌に対してパクリタキセル(175mg/m^2 3週ごと)＋ラパチニブ(1,500mg/日)併用群とパクリタキセル単独投与群との比較においてラパチニブ併用群のほうがHER2陽性乳癌に関して無進行期間(8.1 vs. 5.8ヵ月)(HR＝0.57，p＝0.011)の延長を認め，タキサン系との併用療法にも期待がもたれる[15]。

また，従来の標準的な化学療法に対して抵抗性であると考えられているcancer stem cellに対するラパチニブの効果も示唆されている[16]。日本を含めた国際共同研究として行われているHER2陽性原発性乳癌に対する術後補助療法としてのラパチニブの有効性に関する臨床試験であるALTTO(Adjuvant Lapatinib and/or Trastuzumab Treatment Optimisation)試験の結果に期待がもたれる。

Ⅳ. 耐性のメカニズム

リンパ節転移陽性乳癌に対する術後補助療法のランダム化比較試験であるNSABP B-28試験の結果から，HER2とc-mycは独立した予後因子であることが示されており[17]，NSABP B-31試験のサブ解析では，HER2とc-mycがともに過剰発現している患者ではトラスツズマブの有効性が高かった。c-mycのアポトーシス誘導機能が活性化されるためではないかと推測されている。PTEN (tensin homolog deleted on chromosome ten) が減少するとPI3/Aktシグナルを活性化させトラスツズマブの効果を減弱させることが報告されており[18)-20)]，PTENの欠損している乳癌ではトラスツズマブとパクリタキセルの併用療法に対する反応性が劣ることが報告されている。

HER2同様PI3K/AktやRas/MAPKシグナル伝達系を介するIGF1R (insulin-like growth factor I receptor) は，HER2との相互作用，いわゆる“cross talk”が存在することが報告されている[21]。トラスツズマブとビノレルビンを使用した術前化学療法の試験ではIGF-1の発現している患者群では奏効率が低く，IGF-1発現とトラスツズマブ治療抵抗性の関係が報告されている[19]。

MUC4 (membrane-associated glycoprotein mucin-4) の過剰発現は宿主の免疫系からの逸脱などに関与することが示唆され，乳癌の予後不良因子の1つと考えられている。MUC4はトラスツズマブのHER2結合部位をマスクすることにより，トラスツズマブ治療抵抗性に関与する可能性がある[22]。

HER2/HER3ヘテロ二量体誘発をブロックする抗体であるペルツズマブはトラスツズマブ耐性乳癌に対しての効果が期待されている。2007年に行われたASCOにおいて第二相試験の結果が発表され，33症例のHER2陽性，トラスツズマブ抵抗性の転移性乳癌に対してトラスツズマブとペルツズマブ併用（3週ごとに投与）し1人がCR (complete response)，5人がPR (partial response) となり，奏効率は18.2%だった。さらに7人(21.2%)が，6ヵ月以上の無進行期間を認め，2008年5月に行われたASCOの追加報告において安全性の確認がされている[23)24)]。また，トラスツズマブに抗微小管エージェントであるDM1を結合させた，trastuzumab-DM1 (T-DM1) の第二相試験の結果が2008年12月のサンアントニオ乳癌学会で報告されている。30症例のトラスツズマブ抵抗性転移性乳癌に対してT-DM1を投与したところ，40% (CR 1症例，PR 11症例) の奏効率が認められた[25]。今後より一層トラスツズマブへの耐性のメカニズムの解明や耐性に対する治療法が発展することが期待される。

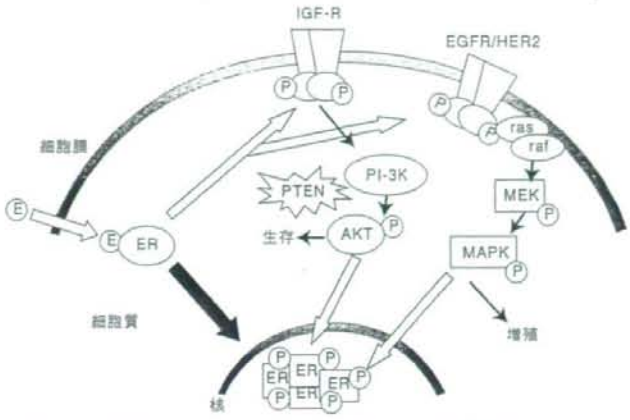

図1．ER受容体とIGF-R，HER2，EGFRのシグナル伝達系クロストーク
（文献[26]より一部改変）

Ⅴ. ホルモン療法と分子標的治療薬

ER受容体とIGF-R，HER2，EGFRのシグナル伝達系は“cross talk”することが知られており（図1）[26]，ホルモン療法に対する抵抗性獲得の原因の1つとして考えられている。アロマターゼ阻害薬とラパチニブを併用することによりエストロゲンによる増殖シグナルが絶たれた後のEGFR/HER2経路の活性化が抑制され，治療効果の向上が期待されている。2008年12月に行われたサンアントニオ乳癌学会においてHER2陽性，ホルモン受容体陽性の閉経後転移性乳癌に対するファーストライン治療としてのレトロゾールとラパチニブの併用の効果が報告されており，レトロゾール単剤に比べラパチニブ併用群で無進行期間（8.2 vs. 3.0ヵ月）(HR=0.71，p=0.019) の延長を認

めた[27]。ホルモン受容体陽性，HER2陰性乳癌に対する併用療法の効果も期待されており，今後の報告が待たれる。

Ⅵ．ベバシズマブ

vascular endothelial growth factor (VEGF)は血管内皮細胞の増殖・遊走に加えて，血管内皮細胞のアポトーシスを阻害し，腫瘍血管新生を維持すると考えられている。また，血管透過性，血管拡張作用，骨髄からの血管内皮細胞補充の増加などの生物学的効果をもたらすとも知られている。ベバシズマブはVEGFに対するヒトモノクローナル抗体(図2)[28]であり，抗癌剤併用治療における効果が知られている転移性乳癌患者462例を対象としたカペシタビンとベバシズマブの併用療法の試験では，カペシタビン単剤群とカペシタビン，ベバシズマブ併用群との比較で無進行期間，全生存期間に有意差を認めなかった。しかし，グレード3以上の有害事象に関して高血圧以外は単剤群，併用群に有意差を認めず，ベバシズマブの安全性が確認された[29]。その後行われたパクリタキセルとの併用試験は転移性乳癌患者722人に対してパクリタキセル単独投与群とベバシズマブ併用群を比較し，無進行期間においてベバシズマブ併用群のほうが有意に無進行期間を改善した(11.8 vs. 5.9ヵ月)(HR＝0.60，p<0.001)[30]。

休薬期間を設けず，低用量の抗癌剤を持続的に投与するメトロノミック療法は一種の抗血管新生療法と考えられており，ベバシズマブとの併用療法が期待されている。メトロノミック療法では休薬期間を設ける治療と比べて，実質および間質のいずれの細胞に対してもアポトーシス促進活性効果があり，治療に対する抵抗性の獲得を減少させることに関して優れているとされている[31][32]。第三相試験において46人の局所進行・再発乳癌患者に対してカペシタビン(500mg/1日3回)＋シクロフォスファミド(50mg/日)＋ベバシズマブ(10mg/kg/2週)の投与を行ったところ，CR 1症例(2％)，PR 21症例(46％)，SD (stable disease) 19症例(41％)であり，奏効率(CR＋PR) 48％，24週以上の治療有用性(CR＋PR＋SD＞24週)は68％であった。さらにこの試験において，治療開始前のcirculating endothelial cells (CECs)が一定以上認められる群では有意に無進行期間の延長が認められており，CECsが血管新生阻害治療のサロゲートマーカーとなりうる可能性を示している[33]。

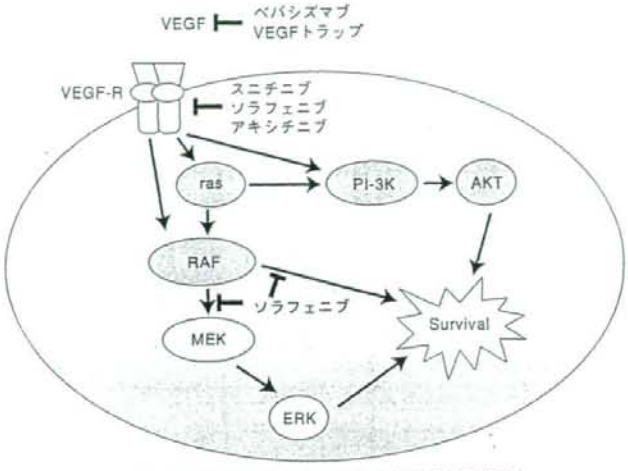

図2．腫瘍増大に関連する血管内皮細胞活性経路
(文献[28]より一部改変)

おわりに

以上のように，次世代を担う分子標的治療薬は，実臨床の場面でもその効果が発揮されており，今後も日々の基礎研究から提供される情報を元にさらに発展していくものと考えられる。同時に薬剤の多様化，複雑化は必須であり，われわれ臨床医の薬剤使用に対するより一層の適切な理解が求められるものと考える。

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The reversal of recurrence hazard rate between ER positive and negative breast cancer patients with axillary lymph node dissection (pathological stage I-III) 3 years after surgery

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Abstract

Backgrounds: Prognostic factors are defined as biological or clinical measurement associated with overall survival and/or disease-free survival. Previous studies have shown that patients with estrogen receptor (ER) positive cancers have a better prognosis than patients whose cancers do not have these receptors.

Methods: This study investigated the assessment of variables in defining prognosis of 742 breast cancer women with pathological stage (pTNM) I-III diagnosed between 1980 and 2005 at the Kyoto University Hospital in Japan, by age, clinical stage (cTNM), pTNM, the numbers of positive lymph nodes (pN), and ER status.

Results: Multivariate analysis demonstrated that pTNM and ER status were the independent prognostic factors for overall survival, and that pTNM and pN were the independent prognostic factors for disease-free survival. For the 0- to 2-year interval, the hazard of recurrence was higher for the ER-negative patients than the ER-positive patients, and beyond 3 years the hazard was higher for ER-positive patients.

Conclusion: The present study confirmed the previous reports which showed favorable prognosis of the patients with lesser pTNM or positive ER status. A reversal of recurrence hazard rate between ER positive and negative breast cancer patients beyond 3 years after operation was detected. The fact may indicate the importance of long term adjuvant hormone therapy for ER positive cancer patients.

Background

A prognostic factor is defined as a biological or clinical measurement that is associated with overall survival and/or disease-free survival [1]. The knowledge of prognosis forms an integral part of the decision-making process in medicine [2]. Moreover, prognostic factors are important in the treatment of cancer to help identify subgroups of patients who may need more aggressive approach to therapy [3]. Further, prognostic factors also play a critical role in designing clinical trial as stratification and allocation factors [4]. Prognostic factors, i.e., those that predict the risk of recurrence or death from breast cancer, include stage, number of positive axillary nodes, tumor size, lymphatic and vascular invasion, the estrogen-receptor (ER) and progesterone-receptor (PR) positivity, and HER2/neu gene amplification [3,5]. We previously reported that the recent advance of the survival rates in breast cancer patients may be due to the rational development of treatment [6]. In order to assess the independent value of variables in defining prognosis, in the present study, we have investigated the survival of 742 breast cancer patients with pathological stage (pTNM) I-III, by the age, clinical stage (*c*TNM), pTNM, the numbers of positive lymph nodes (pN) and ER status.

Methods

Patients

742 female beast cancer patients aged between 21 and 80 with stage I-III of pTNM were selected from the patients treated at Kyoto University Hospital in Japan from 1980 to 2005. Based on the section 2 in chapter 1 of Japanese ethical guidelines for epidemiological research http:www.niph.go.jp/english2/english_ver/ethical-gl/guide_lines.htm, this study was exempt from ethical approval under Japanese law and guidelines. Moreover, all treatments for breast cancer were undertaken with informed consent and consents were also taken to confirm cancer diagnosis. These patients underwent surgery with axillary lymph node dissection. The operation methods were classified into three groups: breast conserving surgery, modified radical mastectomy, and standard radical mastectomy. All the patients with breast conserving surgery received radiation therapy. Staging of *c*TNM and pTNM was evaluated according to UICC stage [7]. Number of lymph node metastasis and ER status of the primary tumors were analyzed by staff members of the Department of Pathology at Kyoto University Hospital. Using immunohistochemistry on the whole series of tumors, they assessed estrogen receptor (ER) status in a standardized way. In our institute, the pathologists routinelyhave examined the ER status of tumors by using the immunohistochemistry since the 1980s. The contents of treatments for breast cancer patients were previously described [6]. According to the years of surgery the patients were grouped into two cohorts: period I (1980–1989) and period II (1990–2005). In period I, modified radical mastectomy with lymph node dissection was included. In this period, breast-conserving surgery was not performed, because it was not recognized to be the prevailing method in Japan. In period II, breast-conserving surgery was the treatment of choice for women with relatively small breast cancers during this past decade in Japan. In our institute, all patients with breast-conversing surgery received radiation therapy. In the treatment stage I, II, IIIA, and operable stage IIIC breast cancer, breast-conserving surgery or modified radical mastectomy with lymph node dissection and with or without breast reconstruction surgery was included. In the treatment of stage IIIB and inoperable stage IIIC breast cancer, systemic chemotherapy, or systemic chemotherapy followed by surgery, with lymph node dissection followed by radiation therapy were included. If necessary, additional systemic therapy such as chemotherapy, hormone therapy, or both were given. Moreover, if necessary, adjuvant therapy such as systemic chemotherapy (*per os* only) with or without hormone therapy (tamoxifen or tremifene) was included. The patients received adjuvant chemotherapy with LH-RH agonist after 2001, cyclophosphamide, epirubicin and 5-fluorouracil (CEF) or Cyclophosphamide, methotrexate and 5-fluouracil (CMF) regimen after 2002, and rational developers such as taxane, trastuzumab, or aromatase inhibitor therapy after 2004.

Statistical analysis

Disease-free survival was defined from the operation day to the identification date of recurrence of cancer or death from any cause, and overall survival was defined from the operation day to death from any cause. Survival curves were estimated with the Kaplan-Meier method. To identify prognostic factors independently associated with the overall survival or disease-free survival and to estimate the hazard ratios, the Cox proportional hazard model was applied. Two-sided $p < 0.05$ was regarded as statistically significant. The statistical analysis was conducted with SPSS version 11.0 statistical software.

Results

Patient Characteristics

Patient characteristics are summarized in Table 1. The medianfollow-uptime of the investigated period in this study was as same as the median follow-up time forsurvivingpatients (5.7 years).

10-year overall survival

The 10-year overall survival rates classified by age, *c*TNM, pTNM, pN, ER status and types of breast surgery are shown in Table 2. Figure 1 shows the overall survival curves in ER-positive and ER-negative patients.

Table 1: Patient characteristics (n = 742)

	number	%
Gender		
female	742	100
male	0	0
Age		
<35 (21–34)	35	4.7
35–54	337	45.4
≥ 55 (55–91)	370	49.9
cTNM stage		
Stage I	197	26.6
Stage II	452	60.9
Stage III	93	12.5
pTNM stage		
Stage I	189	25.5
Stage II	397	53.5
Stage III	156	21.0
pN		
pN0	422	56.9
pN1	189	25.5
pN2	88	11.9
pN3	43	5.8
ER status		
negative	290	39.1
positive	452	60.9
Breast surgery		
Breast conserving surgery	305	41.1
Modified radical mastectomy	429	57.8
Standard radical mastectomy	8	1.1

10-year disease-free survival

The 10-year disease-free survival rates classified by age, *c*TNM, pTNM, pN, ER status and types of breast surgery are shown in Table 3. The approximate 10-year disease-free survival between ER positive and negative patients was reversed (Figure 2). According to age, *c*TNM, pTNM and pN, the reversal of disease-free survival was not detected in the present study (Table 3).

Estrogen receptor status

Because beyond 10 years hazard had increased statistical errors, we investigated the annual hazard of recurrence until 10 years after operation. For the 0- to 2-year interval, the hazard of recurrence was higher for the ER-negative patients than the ER-positive patients, and beyond 3 years the hazard was higher for ER-positive patients (Figure 3). Figure 4 shows that the overall survival of ER-positive cancer patients was increased by adjuvant hormone therapy ($p = 0.009$). Moreover, among 452 ER-positive cases, at 1 year after surgery, the hazard of recurrence was higher for the patients with adjuvant hormone therapy than the patients without adjuvant hormone therapy, but between 2 and 4 years, the hazard was higher for the patients without adjuvant hormone therapy (Figure 5).

Prognostic factor analysis

Age (<35; 35–54; ≥ 55), *c*TNM (stage I-III), pTNM (stage I-III), pN (pN0, pN1, pN2, pN3), ER status (negative, positive, unknown), and types of breast surgery (breast conserving surgery, modified radical mastectomy, radical mastectomy) were analyzed as potential prognostic factors by the Cox proportional hazard model. Both univariate analyses to determine prognostic factors associated with overall survival and disease-free survival that the features with $p < 0.05$ were 5 features: *c*TNM, pTNM, pN, ER status, and type of surgery (Table 2 &3). The important prognostic factor associated with overall survival determined by multivariate analyses with backward variables selection were 2 features: pTNM and ER status (Table 4). The important prognostic factor associated with disease-free survival determined by multivariate analyses with backward variables selection were two features: pTNM and pN (Table 4).

Discussion

Tumor staging systems provide information about extent of disease that can be used to guide treatment recommendations and provide estimates of patient prognosis. It is well known that pathological stage is the most significant independent prognostic factor for determining survival in breast cancer [8]. Our study documents the fact that pathological stage is the independent prognostic factor for both overall survival and disease-free survival.

Many studies have shown that women with ER positive cancers have a better prognosis than patients whose cancers do not have this receptor [9,10]. In this study cohort, ER status were the independent prognostic factors for overall survival by the multivariate Cox regression analysis, but ER status did not affect disease-free survival (Table 3 &4). Nomura et al. [11] previously reported that in a retrospective multicenter study to investigate the ER status in primary breast cancer with patient prognosis, 3,118 patients with operable breast cancer (stages I-III) were investigated from ten hospitals in Japan who underwent surgery from October 1972 to December 1982, and that Cox's multivariate analysis showed that overall survival, but not disease-free survival was affected by ER status. They speculated the possibility that this was due to the longer postrelapse survival in patients with ER-positive cancer based on the effectiveness of endocrine treatment. Preceding paper has reported that the patients of positive ER status enjoyed benefits from the recent development of breast cancer treatments [6]. In fact, the present study showed that the overall survival of ER-positive cancer patients was increased by adjuvant hormone therapy (Figure 4).

Hortobagyi et al. [12] previously reported that the disease-free survival in estrogen receptor (ER) positive and/or pro-

Table 2: The 10-year overall survival rates and univariate Cox regression analysis

Factors	overall survival rates		Hazard ratio		Log-rank test p-value
	10-year (%)	95% CI[a]		95% CI [a]	
Age					
< 35	69.6	57.7–81.5	1.00	-	0.30
35–54	78.1	75.2–81.0	0.69	0.30–1.59	
≥ 55	73.4	69.9–77.0	0.90	0.39–2.08	
cTNM					
Stage I	85.7	81.3–90.0	1.00	-	<0.001
Stage II	75.8	73.1–78.5	2.32	1.31–4.09	
Stage III	54.5	46.9–62.0	4.85	2.55–9.22	
pTNM					
Stage I	89.4	85.8–93.1	1.00	-	<0.001
Stage II	81.7	79.1–84.4	2.10	1.10–4.03	
Stage III	46.4	40.8–51.9	7.77	4.08–14.81	
pN					
pN0	86.7	84.2–89.1	1.00	-	<0.001
pN1	76.4	72.1–80.7	1.74	1.08–2.82	
pN2	46.6	39.7–53.4	5.25	3.34–8.27	
pN3	38.2	26.9–49.5	5.34	3.01–9.47	
ER status					
Negative	71.0	67.7–74.3	1.00	-	0.012
Positive	79.5	76.5–82.5	0.63	0.44–0.91	
Breast surgery					
Breast conserving surgery	76.1	71.2–81.0	1.00	-	0.093
Modified radical mastectomy	76.2	73.7–78.7	1.31	0.84–2.04	
Standard radical mastectomy	19.1	2.29–35.8	4.09	1.57–10.64	

[a] CI: Confidence Interval.

gesterone receptor (PgR) positive patients was higher than that in ER/PgR negative patients until 5 years after administration of the state-of-the-art adjuvant therapy, however, the disease-free survivals between these groups was reversed after 5 years. Saphner et al. [13] reported that compared with ER negative patients, ER positive patients had lower annual hazard of recurrence until around 3.5 years after surgery, but thereafter higher. In the present study, Figure 3 shows that a positive ER status was associated with a lower hazard of recurrence in the first 2 years after surgery, but a higher hazard of recurrence from years 3 to 10. [14]. Results from the EBCTCG meta-analysis of systemic treatment of early breast cancer by hormone, cytotoxic, or biologic therapy methods in randomized trials involving 144,939 women show a highly significant advantage of 5 years versus 1 to 2 years of tamoxifen with respect to the risk of recurrence [14]. In the present study, in ER-positive cases, between 2 and 4 years after surgery, the hazard of recurrence of patients without adjuvant hormone therapy was higher than the patients with adjuvant hormone therapy (Figure 5). It is noteworthy that this observation emphasizes the importance of adjuvant hormone therapy for ER positive cancer patients beyond 3 years after operation. Moreover, comparing with the 10-year survival rate between ER-positive patients with or without hormone therapy and ER-negative patients (Figure 1 &4), the survival rate between ER-positive patients without hormone therapy and ER-negative patients was similar, but the adjuvant hormone therapy led about 13%

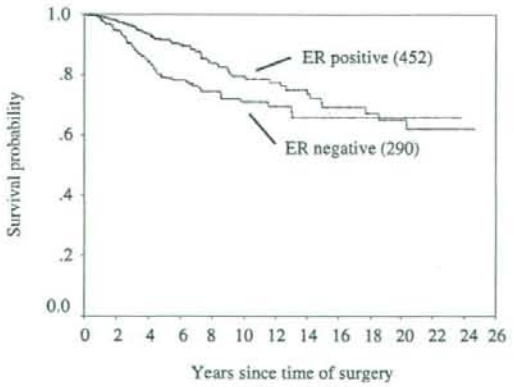

Figure 1
Overall survival curves in ER-positive and ER-negative patients. (Number) = number of patients. $p = 0.012$.

Table 3: The 10-year disease-free survival rates and univariate Cox regression analysis

Factors	disease-free survival rates		Hazard ratio		Log-rank test p-value
	10-year (%)	95% CI [a]		95% CI [a]	
Age					
< 35	47.0	33.3–60.7	1.00	-	0.49
35–54	59.0	55.7–62.4	0.91	0.50–1.64	
≥ 55	63.1	59.4–66.7	0.90	0.50–1.63	
cTNM					
Stage I	72.4	67.3–77.6	1.00	-	<0.001
Stage II	60.9	58.0–63.9	2.05	1.45–2.91	
Stage III	31.5	24.8–38.2	5.03	3.36–7.52	
pTNM					
Stage I	81.7	77.4–85.9	1.00	-	<0.001
Stage II	67.5	64.4–70.5	2.18	1.47–3.24	
Stage III	17.7	13.1–22.4	7.66	5.13–11.43	
pN					
pN0	76.6	73.8–79.5	1.00	-	<0.001
pN1	56.4	51.6–61.2	1.85	1.36–2.53	
pN2	15.5	10.3–20.7	5.75	4.22–7.83	
pN3	26.8	16.6–37.1	4.88	3.25–7.32	
ER status					
Negative	59.8	56.4–63.2	1.00	-	0.183
Positive	60.0	56.6–63.4	0.83	0.63–1.09	
Breast surgery					
Breast conserving surgery	59.1	54.1–64.0	1.00	-	0.007
Modified radical mastectomy	61.5	58.7–64.3	1.14	0.86–1.52	
Standard radical mastectomy	ND[b]	-	3.34	1.76–6.33	

[a] CI: Confidence interval, [b] ND: not determined.

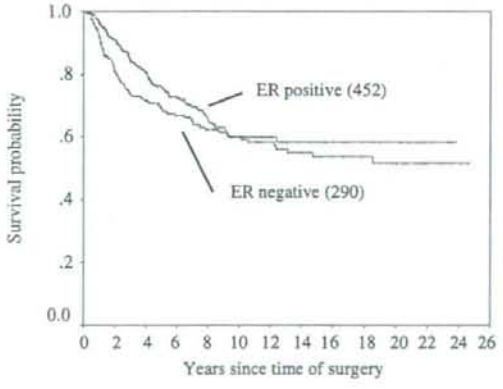

Figure 2
Disease-free survival curves in ER-positive and ER-negative patients. (Number) = number of patients. *p = 0.18.*

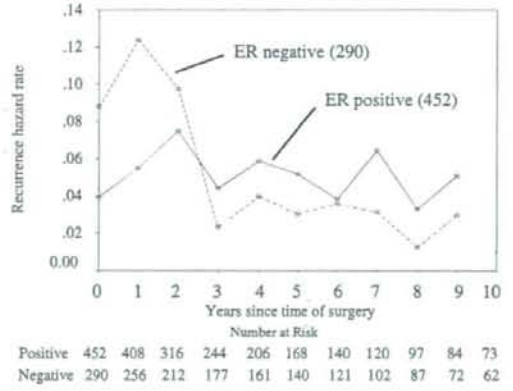

Figure 3
Annual hazard of recurrence of patients separated by ERstatus. (Number) = number of patients.

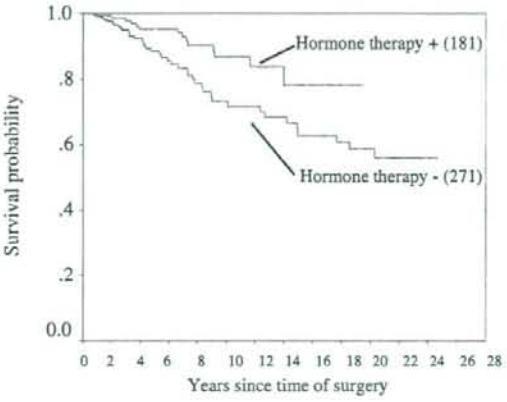

Figure 4
Overall survival curves in ER-positive patients with and without adjuvant hormone therapy. (Number) = number of patients. $p = 0.009$.

survival gains. Therefore, this fact also suggests adjuvant hormone therapy may have more important roles in the treatment. In addition, the disease-free survival at 10 years after surgery between ER positive and negative patients was reversed (Figure 2). This may be related to the fact that the percentage of number of patients who received adjuvant hormone therapy in ER positive patients between 1980 and 1991 (11/84: 13%) was smaller to that between 1991 and 2005 (170/368: 46%), because of reasons including poor understanding of modern treatment for adjuvant chemotherapy, the cost for drugs, and so on. On

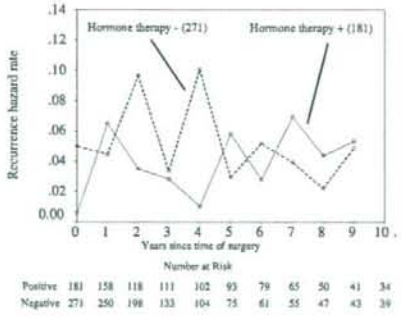

Figure 5
Annual hazard of recurrence of ER-positive patients separated by adjuvant hormone therapy. (Number) = number of patients.

Table 4: Multivariate Cox regression analysis for overall survival and disease-free survival.

Factors	Overall survival		
	Hazard ratio	95% CI [a]	p-value
pTNM			
Stage I	1.00	-	
Stage II	2.05	1.07–3.94	0.03
Stage III	8.09	4.24–15.43	<0.001
ER status			
Negative	1.00	-	
Positive	0.57	0.40–0.82	0.002
Factors	**Disease-free survival**		
	Hazard ratio	95% CI [a]	p-value
pTNM			
Stage I	1.00	-	
Stage II	1.87	1.12–3.11	0.017
Stage III	3.72	1.59–8.70	0.002
pN			
pN0	1.00		
pN1	1.49	1.01–2.20	0.044
pN2	2.47	1.14–5.34	0.022
pN3	1.91	0.83–4.39	0.129

[a] CI: Confidence interval

the other hand, the current recommendation is that adjuvant tamoxifen be discontinued after 5 years in all patients as current standard therapy, because there was a trend toward a worse outcome associated with a longer duration of treatment [15]. Further analyses may be needed to clarify the optimal duration of adjuvant hormone therapy in operated breast cancer patients.

Traditional prognostic factors, i.e., those that predict the risk of recurrence or death from breast cancer, include number of positive axillary nodes [3]. It has been reported that the pN is the most important prognostic factor affecting disease-free survival and overall survival in operable breast cancer patients [2]. However, our study suggested that pN is the independent prognostic factor for disease-free survival, but not for overall survival. The patients with axillary lymph node metastasis have received chemotherapy, hormonal therapy or both. Over the past 20 years, various systemic adjuvant therapies have been studied to improve survival [6]. Therefore, there may be a possibility that the other factors such as these therapies may affect the overall survival more stronger than pN, although further investigations are needed to clarify this matter.

The univariate Cox regression analysis for overall survival and disease-free survival demonstrated that the hazard ratio of patients with breast conserving surgery was lower

than that of patients with standard radical mastectomy (Table 2 &3). This fact suggests that breast conserving surgery with radiation therapy may provide not only cosmetic benefit but also better prognosis, although chronological change of breast cancer treatments may affect the survival rates.

In conclusion, the present study presented the data of the long term survival of pathological stage I-III patients with breast cancers at our institution. For the 0- to 2-year interval, the hazard of recurrence was higher for the ER-negative patients than the ER-positive patients, and beyond 3 years the hazard was higher for ER-positive patients. Additionally, disease free survival 10 years after operation was reversed between ER-positive and negative patients. Therefore, the fact may indicate the importance of long term adjuvant hormone therapy for ER positive cancer patients.

Abbreviations

cTNM: clinical stage; ER: estrogen receptor; pTNM: pathological stage; pN: positive lymph nodes.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TI and MF designed this study. MU, KY, HK, TI collected and assembled the data. TI organized the data. TK, TN, ST, TI and MF contributed to the statistical analyses and interpretations. TK, TI, MT and MF contributed to writing and finalizing of the manuscript. All authors read and approved the final manuscript.

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Adipogenesis Induced by Human Adipose Tissue–Derived Stem Cells

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Adipose tissue–derived stem cells (ASCs), including preadipocytes, may play an important role in *de novo* adipogenesis and are expected to be a useful external source of cells for adipose tissue engineering. In this study, we examined *in vivo* adipogenesis up to 24 weeks after implantation, induced by human ASCs that were isolated from adipose tissues and expanded *in vitro*. ASCs proliferated *in vitro* in the presence of basic fibroblast growth factor (bFGF), and the number of cells increased by more than 1000-fold at the fourth passage. The ability to differentiate into mature adipocytes was maintained up to the third passage. We incorporated designated numbers of third-passage–expanded cells into a type I collagen scaffold and implanted them into the back of nude mice with or without controlled-release bFGF. After the implantation of 2×10^6 ASCs with controlled-release bFGF, the greatest cross-sectional surface area of adipose tissue in the scaffold was 1.19 mm^2 at 12 weeks and 2.14 mm^2 at 24 weeks. About 2×10^6 ASCs with controlled-release bFGF was the best condition for total adipogenesis. Immunohistochemical analysis with antihuman vimentin antibody showed that the area of human-origin adipose tissue was maximum in the group with 8×10^6 ASCs incorporated in a scaffold at both 12 and 24 weeks. The amount of human-origin adipose tissue increased in all groups with implanted ASCs from 12 to 24 weeks. Only trace of human-origin adipose tissue was observed in other groups implanted ASCs. Our results show that human ASCs not only function as progenitor cells for *in vivo* adipogenesis, but also induce *de novo* adipogenesis for long period.

Introduction

BREAST CANCER IS THE MOST COMMON CANCER in women, and surgery remains one of the main treatments. Breast surgery results in deformity of the breast and negatively affects patients' quality of life. Perforator flaps or silicone implants have been used for breast reconstruction, but each has advantages and disadvantages. Several trials of autologous adipose tissue transplantation for breast reconstruction resulted in a 40–60% reduction in adipose tissue volume because of insufficient vascularization.[1–6]

Recent studies in tissue engineering indicate that cell proliferation requires an appropriate cell source, scaffold, and microenvironment, including growth factors.[7,8] The ideal cell source for tissue engineering must have self-renewal capability and immunocompatibility.[9] Mesenchymal stem cells (MSCs) isolated from bone marrow stroma can differentiate into adipogenic, osteogenic, myogenic, and chondrogenic lineages. However, the procurement of cells from bone marrow that are suitable for clinical use has several drawbacks, including severe pain, morbidity, and a low yield.[10] Adipose tissue–derived stem cells (ASCs) can be isolated from collagenase digests of adipose tissue. Various kinds of term have been used for this cell population, for example, adipose-derived stem/stromal cells, adipose-derived adult stem cells, preadipocytes, processed lipoaspirate cells, and adipose mesenchymal stem cells. The International Fat Applied Technology Society reached a consensus to adopt the term "adipose-derived stem cells" to identify the isolated, plastic-adherent, multipotent cell population.[11] ASCs also can differentiate into adipogenic, osteogenic, myogenic, and chondrogenic lineages similar to MSCs.[12] Moreover, a comparison of MSCs and ASCs from the same patient showed no significant differences in the yield of adherent stromal cells, growth kinetics, cell senescence, multilineage differentiation capacity, or gene transduction efficiency.[13] Gene array analysis revealed that less than 1% of genes were differentially expressed between ASCs and MSCs. ASCs were superior to MSCs with respect to maintenance of proliferating ability.[14] The fraction of preadipocytes contributing to adipogenesis

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differs among species and ages. In humans the fraction is 1% in children and less than 0.1% in adults.[15] Human adipose tissue is abundant and can be obtained more safely and easily under local anesthesia or at breast surgery than bone marrow cells.

During differentiation into mature adipocytes, preadipocytes express several types of extracellular matrix (ECM) proteins, including fibronectin, laminin, and types I, III, IV, V, and VI collagen. A fibronectin network develops initially, and a type I collagen network is formed last. These ECMs allow preadipocytes to differentiate into mature adipocytes.[16] In response to adipogenic stimulation, ASCs form tissue sheets harboring lipid-filled adipocytes embedded into an abundant human ECM.[17] *In vivo* adipogenesis depends on the type of ECM.[18] Type I collagen has been widely used as a scaffold for adipose tissue engineering[19–22] because of its porous structure. Preadipocytes readily adhere to and grow in type I collagen scaffolds.[23] An *in vitro* study reported a higher rate of adipogenic differentiation with type I collagen than with fibronectin.[24]

In vitro proliferation of ASCs is enhanced by basic fibroblast growth factor (bFGF).[25] Although it remains controversial whether bFGF has direct adipogenic activity,[26–29] bFGF has been shown to promote adipogenesis *in vitro* and *in vivo*.[30–32] We found that the controlled-release bFGF more effectively promoted adipose tissue regeneration than aqueous bFGF.[20] We have reported that controlled-release 1 μg of bFGF/site was the most effective concentration on adipogenesis 6 weeks after implantation into nude mice, and a high dose of bFGF caused the inflammatory response in the collagen scaffold.[20]

In a previous study, we implanted up to 5×10^5 human ASCs into nude mice and obtained newly formed adipose tissue 6 weeks after implantation. However, quantitative and qualitative differences in the implanted ASCs were not examined, and not examined for long time. The present study was therefore investigated the optimal passage number *in vitro* and the effects of the number of implanted ASCs on adipogenesis *in vivo* over the period up to 24 weeks.

Materials and Methods

Human ASCs

This study was approved by the Kyoto University ethics committee. Informed consent was obtained from all patients. All patients were women 29–76 years of age. Samples of human adipose tissues were obtained as surgical waste tissue at breast surgery in Kyoto University Hospital (Kyoto, Japan). Donor samples for *in vitro* study were obtained from patients 30–76 years of age with a mean age of 58.6 years ($n = 14$). We allocated seven donors individually to with or without bFGF treatment group. There was no significance of age between with and without bFGF treatment groups. Donor samples for *in vivo* study were obtained from patients 29–70 years of age with a mean age of 50.8 years ($n = 8$). All donor samples were divided equally. ASCs were isolated from the adipose tissue samples as soon as possible after resection by a modification of the procedure described by Bjorntorp *et al.*[33] Briefly, the adipose tissue samples were washed with phosphate-buffered saline (PBS, pH 7.4) to remove blood cells, minced, and digested with collagenase (2 mg/mL; Wako Pure Chemical, Osaka, Japan) in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) and 20 mg/mL bovine serum albumin at 37°C for 40 min while shaking. The digested tissue was suspended in DMEM:Nutrient Mixture F-12(Ham) (1:1) (DMEM/F-12) containing 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (0.1 mg/mL) (basal medium). The suspension was filtered through a 250-μm nylon mesh and centrifuged at 400 *g* for 10 min at 20°C. The sediment was suspended in basal medium, placed in 10-cm tissue culture dishes (Falcon; Falcon, New York, NY), and cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37°C for 1 day. The dishes were gently washed with PBS to remove nonadherent cells and filled with the basal medium and cultured until the adherent ASCs became confluent (P0:Passage0). Then ASCs were detached with 1% trypsin-EDTA solution (Sigma, St. Louis, MO).

Proliferation ability of ASCs

ASCs were suspended in the basal medium, placed in 10-cm tissue culture dishes or 96-well microtiter plate (Falcon) at the density of 1.0×10^4 cells/cm^2, and cultured with 100 ng/mL bFGF or without bFGF for 1 week.[34] To evaluate proliferative activity, viable cells in each dish were counted by the trypan blue dye exclusion method at the end of culture. These numbers represent the proliferation of undifferentiated ASCs. The proliferation of ASCs was also evaluated by MTT assay.[35] Using a commercially available kit for MTT assay (Chemicon International, Temecula, CA), we spectrophotometrically measured the absorbance of the solution mixture in each well at 570–630 nm.

Differentiation ability of ASCs

ASCs suspended in basal medium were placed in 24-well plates (Falcon) at a density of 1.0×10^5 cells/cm^2 and cultured for 1 day. The medium was then changed to DMEM/F-12 medium (1000 μL/well) containing 0.05 μM insulin, 0.2 nM 3,5,3-triiodothyronine, 100 nM transferrin, 17 μM calcium pantotenate, 33 μM biotin, and 100 nM dexamethasone (ITT medium)[36] and cultured for 21 days. To evaluate adipogenic differentiation of ASCs, glycerol-3-phosphate dehydrogenase (GPDH) activity was measured using a commercially available kit (GPDH activity measurement kit, JFL003; Hokudo, Hokkaido, Japan).[37] ASCs were washed twice with PBS and homogenized in the buffer solution included with the kit, using a handy sonic homogenizer (UR-20; Tomy Seiko, Tokyo, Japan) on ice. After mixing, the absorbance of the solution mixture was spectrophotometrically measured at 340 nm.

Materials

We prepared a disc form of type I collagen scaffold (diameter, 20 mm; height, 2.5 mm) and gelatin microspheres (isoelectric point, 5.0), as described in our previous report.[20,38] An aqueous solution of human recombinant bFGF was kindly supplied by Kaken Pharmaceutical, Tokyo, Japan. Other chemicals were purchased from Wako Pure Chemical Industries, Kyoto, Japan, and used without further purification. To prepare controlled-release bFGF, 2 mg of gelatin microspheres was swollen with an aqueous solution of bFGF (20 μL, containing 1 μg of bFGF) and allowed to stand at 37°C for 1 h.

Implantation of ASCs

Animal experiments were reviewed by the Committee on the Ethics of Animal Experiments (Faculty of Medicine,

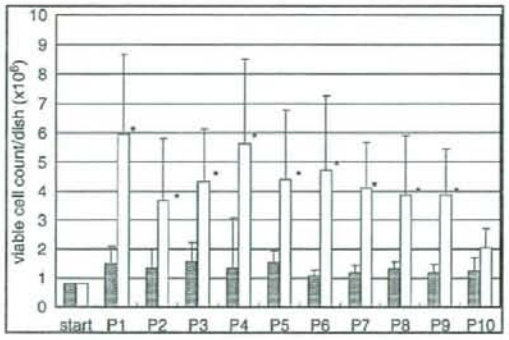

FIG. 1. Proliferation of ASCs *in vitro* assessed on the basis of the viable cell count. The number of viable ASCs per 10 cm of the tissue culture dish after culture with 100 ng/mL bFGF (open bars) or without bFGF (closed bars) for 1 week was measured by trypan blue dye exclusion assay. *: $p < 0.05$ versus without bFGF group.

Kyoto University, Kyoto, Japan) and were carried out in accordance with the Guidelines for Animal Experiments of the Faculty of Medicine, Kyoto University.

The designated number of third-passage ASCs (0: A group and B group; 5×10^5: C group and D group; 2×10^6: E group and F group; 8×10^6: G group and H group) were incorporated into the collagen scaffolds with (B, D, F, and H groups) or without controlled-release bFGF (A, C, E, and G groups), and implanted subcutaneously into the back of 6-week-old female BALB/c nude mice (Shimizu Laboratory Supply, Kyoto, Japan) under general anesthesia. Twelve-week group consisted of five mice. Twenty-four-week group consisted of three mice. Twelve and 24 weeks after implantation, the mice were euthanized with an overdose of anesthesia, and the implanted sites including the skin (approximately $2\times2\,cm^2$) were carefully removed for subsequent histological examinations.

De novo *adipogenesis and human ASC–derived adipogenesis*

One half of each tissue specimen was fixed in 10% neutralized formalin solution and embedded in paraffin. Sections (thickness, 2 μm) of the specimens were stained with hematoxylin and eosin (H-E). The other half of the specimen was frozen, and sections were stained with oil red O to confirm the presence of mature adipose tissue. Paraffin sections were stained with a monoclonal antibody against human vimentin (mahv, clone V9, Code Nr. M 0725 Lot 057; DAKO, Glostrup, Denmark). The antibody was used at a dilution of 1:25. The positive control was human adipose tissue, and the negative control was murine adipose tissue. Adipose tissue area of the scaffolds and the human vimentin–positive area were measured and analyzed with the computer program Image-Pro Plus (Media-Cybernetics, Bethesda, MD). We took pictures of H-E sections with Axio Vision (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) software, and opened these pictures with Image-Pro Plus. We measured the area of scaffolds and newly formed adipose tissue with the manual measurement function. Scaffolds were stained with H-E, but lipid droplets were not stained. We excluded fibroblastic capsules around the scaffolds. Morphologically, collagen scaffolds have a net-like structure, adipose tissue has a granulated structure, and fibroblastic capsules have a layered structure. The average area of the scaffolds was 2.14 mm^2 and did not differ significantly among the groups.

Statistical analysis

The Mann–Whitney U-test (Microsoft Exel, Statcel2) was employed for statistical analysis, and $p < 0.05$ was considered to indicate statistical significance.

Results

Proliferative activity of ASCs

The proliferative activity of the ASCs *in vitro* was retained through the 10th passage as assessed by the viable cell count (Fig. 1) and MTT assay (Fig. 2). The proliferative activity of the ASCs was 1.6–4.0-fold higher in the presence of bFGF than in the absence of bFGF (Fig. 1). The number of ASCs increased by more than a 1000-fold at the fourth passage of ASCs cultured with bFGF. Statistically significance was seen in the presence of bFGF (Figs. 1 and 2). There was no correlation between the proliferative activity of the ASCs and age of donors.

Differentiation of ASCs to mature adipocytes

Differentiation of ASCs to mature adipocytes as assessed by GPDH activity assay was observed at all passages (Fig. 3). The extent of differentiation of ASCs in the presence of bFGF was greater than that in the absence of bFGF from the first to third passages, and decreased from the fourth passage onward. From first to third passage, differentiation of ASCs in the presence of bFGF was significantly greater than any other groups. Open and closed bars at start show intrinsic GPDH. There was no correlation between the extent of differentiation and age of donors.

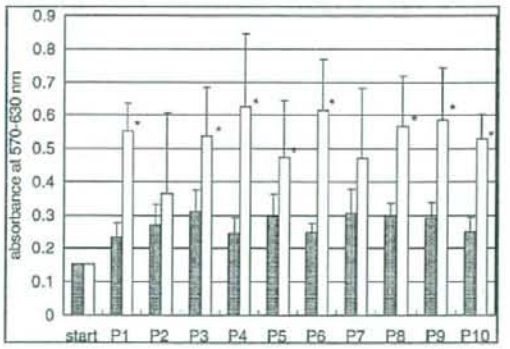

FIG. 2. Proliferation of ASCs *in vitro* as assessed by MTT assay. Proliferation of ASCs in 96-well microtiter plates cultured with 100 ng/mL bFGF (open bars) or without bFGF (closed bars) for 1 week was measured by MTT assay. *: $p < 0.05$ versus without bFGF group.