と消化管が最低2 cmは両者が離れていないと，消化管に障害を起こす．難治性の潰瘍や出血を起こすことがある．胃，十二指腸，大腸との位置関係が重要となってくる．また肝門部の照射も注意が必要である．腸管と胆管の問題がつきまとうことが今後考慮しなければならない課題である．これらは相対的禁忌の範疇であると考えている．

6 陽子線治療の現状と今後

2006年10月現在で，陽子線治療が施行されているところは，世界で18施設，本邦で6施設である．炭素線は，世界で3施設，本邦で2施設稼働しているのが現状である．全世界で陽子線治療では，42,766名，重イオンで4,520名の患者さんが治療を受けている[23]．この中でもHCCに対する治療は圧倒的に本邦で施行されている．

HCCに対する重荷電粒子線治療として現在，HCCに対する陽子線治療症例数と，観察期間の長さにおける臨床治験の蓄積は筑波大学の施設が最大規模である[12]．これについで，本邦においても，国立がんセンター東病院[13]，米国ロマリンダ大学も報告をしている[27]．兵庫県の播磨公園科学都市に兵庫県立粒子線医療センター，静岡県立がんセンターなどで陽子線治療専用施設がすでに稼働している．最も新しい施設としては，福島市に民間病院として初めて南東北がん陽子線治療センターが2008年10月より稼働している．

陽子線は，最も実用に近い新しい放射線治療というのが大方の一致した見解である．陽子線治療は現在まだ保険適応になっていないため，治験終了施設においては，250万～300万円前後が全額自己負担となっているのが現状である．重荷電粒子線治療の中でも陽子線照射療法は設備などのコストパフォーマンスの面からも進歩，改良がみられつつある．専用加速器に小型化などにより，コストのかかる重イオン治療に比べ，腫瘍を取り扱う専門病院での深部臓器癌治療も将来可能になることは十分に予想されるところである．しかし一方，重粒子線に関しても，小型化，低価格化の検討がなされだしている．今後，重荷電子療法の位置付けをどのようにするかこれも，重要な課題である．

7 まとめ

HCCに対する放射線治療は従来の照射方法よりも，陽子線，重粒子線は，効果的かつ安全で耐用性に富み，さらには繰り返し可能であることが第二相試験で示された．外科手術同様，根治目的の治療選択肢の一つとして用いられる可能性があり，また腫瘍径や局在，血流，門脈塞栓，合併症などの条件に制限が少なく，HCCに対して幅広い適応を有すると考えられる．しかし，コストや保険診療，RCTによる科学的根拠に基づく有効性の確立など，いくつかの課題も抱えており，今後明確にする必要があろう．

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特集　肝・胆道・膵がん治療の動向―最新のエビデンス

肝がん
3)放射線療法の位置づけ*

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Key Words : proton beam, local control, QOL, Bragg peak, PVTT

はじめに

本邦における第17回全国原発性肝癌追跡調査報告によると肝癌における治療に関しては，原発性肝細胞癌(hepatocellular carcinoma：HCC)の外科手術以外の治療法の状況は，局所療法31.2%〔percutaneous ethanol injection(PEI)21.4%，microwave coagulation therapy(MCT)11.6%，radiofrequency ablation(RFA)65.8%〕，塞栓療法29.6%であり，局所療法と塞栓術が主流を占める．これらに比し放射線照射療法はわずか1.3%である[1]．

現在，HCCに対する多くの治療法は，それぞれ適応と限界がある[2]．肝臓は放射線への耐容性が低く，従来は肝細胞癌に対する放射線療法が試みられ，有効性も報告されてきたが，照射による肝機能低下のため積極的な治療法とはならなかった．近年，放射線治療は限局部位への線量集中技術の進歩とともに適応も拡大され，選択的腫瘍照射法として従来の放射線を利用したconformal radiotherapy，体幹部定位放射線治療による放射線治療の有効性も報告されている[3)~9)]．さらに，新しい陽子線，炭素線などの重荷電粒子線照射療法など本邦における肝細胞癌に対する放射線治療の進歩は著しく，有効性を示す成績も散見される[10)~14)]．

このように今日放射線療法に関し，残念ながら科学的根拠に基づく多施設無作為試験(CRT)報告はないが，HCCの集学的治療の一環としての放射線療法の有効性は，現段階ではphase II試験として有効性を示唆する報告により支持されている．

本稿においては，これら現在の肝癌に対する放射線治療の現状から，特に放射線治療の最先端の重荷電粒子線治療の現状を示す．放射線同位元素を用いた内照射療法は，本邦で施行される可能性は少ないと考えられるため割愛し，今後の本治療法の占める位置づけを明らかにしたい．

肝細胞癌に対する従来の放射線外部照射法

HCCに対する局所治療法として放射線照射療法は試みられてきた．しかし，外部照射である全肝照射が中心であったために，肝不全出現のため有効性が示唆されながらも，なかなか積極的な治療法としては確立されてこなかった．

HCCに対する光子線治療の外部照射は年代を

* The clinical significance of radiotherapy for hepatocellular carcinoma.

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追ってみると，高良ら[15]によれば30～50Gyの照射にて，病理組織学的に一部に腫瘍細胞の残存を認めたことからこの線量では根治性の面からは不十分とした．さらに，Ohtoら[16]は，39例のHCC患者にlinacを30～50Gy照射し，直径5 cm以下のHCCの場合，縮小率は90%に認められ，5 cm以上では，50%以上の腫瘍縮小率が約60%に認められるという良好な成績を報告している．吉川ら[17]は，肝動脈塞栓療法とリニアックX線約50Gyを局所照射併用し，良好な成績を報告している．野ッ俣ら[18]は，VP3以上の進行肝細胞癌に対して，50Gy以上照射し縮小効果を90%に認めたと報告した．同様の報告は近年においてもなされている．

このような従来の外部肝照射法の最大の難点は，正常組織への照射による副作用である．非肝硬変(liver cirrhosis：LC)例での全照射の耐容線量は30～40Gyとされ，一方HCCに対する根治線量は50Gy以上必要とされる．したがって，従来の外部照射法ではHCCに対し抗腫瘍効果を得るのに十分な線量を局所に照射しえず，安全かつ確実な治療体系にはならなかった．

肝細胞癌に対するconformal radiotherapyと定位放射線治療

近年CT検査の進歩により，3次元的位置情報をもとにコンピューター上においてシミュレーションをし，最適な照射方向，範囲，線量を考慮し行うことが可能となった．原体照射，多門照射法，回転照射法などの照射法の工夫や，呼吸位相同調照射装置の開発[19]など，より限局した部位への高線量照射が試みられている．Conformal radiotherapyは，回転する線源に連動し照射野を変化させ，いろいろな方向から腫瘍の3次元的形態にあわせて照射する方法である．最近はCTを利用し，切除不能HCCに照射効果があったという報告も散見され照射治療計画により，安全にかつ正確に治療できるとしている[3)~7)]．一方，定位放射線治療(stereotactic multiple arcs radiotherapy)とは多数の角度から，細い高エネルギーX線の線束を腫瘍に集中させ照射する照射術である[8)9)]．最近，本邦ではTakedaら[8]が，短期間の定位放射線治療が，HCCの良好な局所制御を得られることを報告している．これらの照射法の利用により良好な成績が得られるようになってきているのが現状である．また，姑息的照射として本照射法の技術も応用され，門脈腫瘍塞栓(portal vein tumor thrombosis：PVTT)や胆管侵襲による閉塞性黄疸などの病態改善を目的として行われ，良好な成績も出されている[20)~25)]．照射野を絞り，ターゲット照射をするconventional radiothepyが近年の従来の放射線を利用したHCCに対する治療の新しい方法である．

最近，定位放射線照射法を改良した新しい，高精度放射線治療統合システムが開発されている．頭部・頸部，脊椎や肺，肝臓，前立腺などの体幹部の腫瘍などに適用可能な高精度放射線治療統合システムである．患者位置決め装置をはじめ，拡大された照射野(22×40cm)を持つ放射線ビーム形成装置を搭載し，より大きな病変部にもピンポイントでの治療が可能となる．このシステムは，放射線量の強弱を調整しながら病変のみに高線量を照射することが可能な，強度変調放射線治療(intensity modulated radiation therapy：IMRT)を実施することが可能である．IMRTとは，コンピュータ制御の特殊照射法であり，腫瘍部分のみに放射線を集中して照射できる画期的な新照射技術である．これによって，従来法では不可能であった理想的な放射線治療が可能となり，腫瘍制御率の向上や合併症の軽減が期待される．今後本治療法による，肝細胞癌に対する成績が蓄積されてくるものと考えられる．

門脈腫瘍塞栓に対する放射線療法

本邦においては，Ohtoら[16]，高良ら[15]はPVTTに対する放射線照射療法の有効性を1980年代にすでに報告している．その後，transcatheter arterial embolization(TAE)との併用での有効性を吉川ら[17]が報告している．本邦においてYamadaらは[25]，3D-CT下conformal radiotherapyにての高線量照射の有効性を報告している．Tazawaら[21]も，経カテーテル的肝動脈化学塞栓術とliniac放射線治療の併用でChild A症例でPVTTに有効であると報告している．Hataら[24]も陽子線照射療法にて，門脈腫瘍塞栓を伴う高度進行肝硬変合併

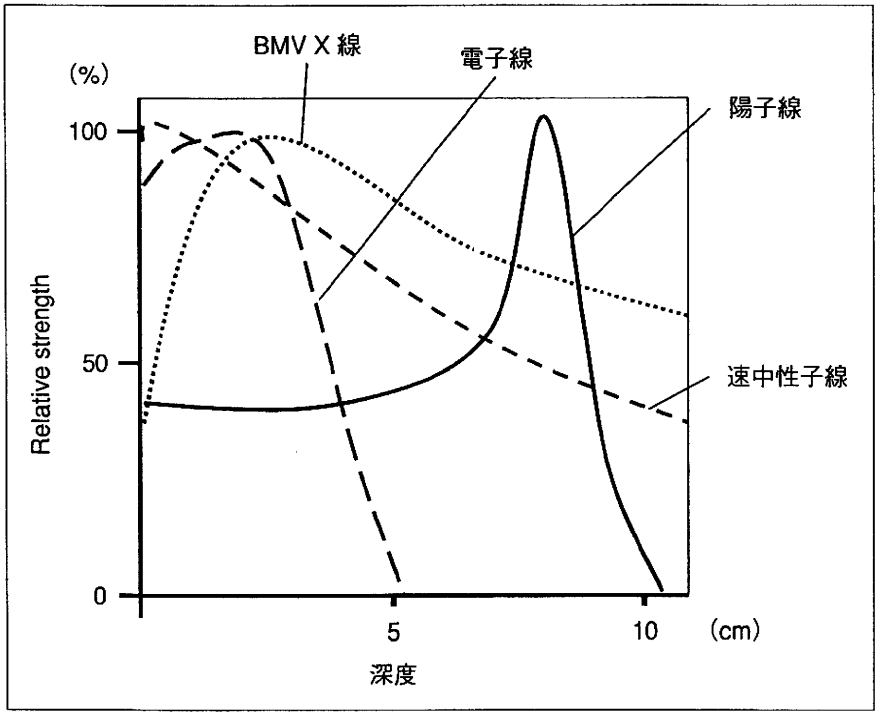

図1 陽子線が持つブラーグ・ピーク特性—狙い撃ちが可能照射—

HCCに照射し，塞栓も縮小し急速に悪化する肝機能障害もとめることが可能と報告している．このように，患者のquality of life(QOL)を損なうことなく，高度進行肝硬変合併HCCの治療が可能となる．

新しい放射線療法—重荷電粒子線治療—

1．粒子線治療の理論的根拠

重粒子とは電子より重い粒子のことを言い，これを加速器で高速にしたものを広義の重粒子と呼ぶ．重粒子線は，さらに非荷電粒子と重荷電粒子の2つに分類される．前者は中性子であり，後者はさらに，陽子，重イオン，p中間子の3つに分類される．新しい放射線療法として現在臨床応用されているものは，重荷電粒子線として陽子線，重イオン(炭素，アルゴン，ネオンなど)があげられる．

陽子は，水素原子から軌道電子をはぎ取った正の電荷を持った粒子(電子の1,836倍の質量を持つ)である．加速器を使い粒子を加速すると高い運動エネルギーを持つ，透過力の大きい電離放射線となる．陽子自身が持つ正の荷電により体内の組織を構成する原子中の電子に力を及ぼし電離，励起をひき起こし，その反作用でエネルギーを失い減速され，最後に速度が0になったところで止まる．このため，陽子の運動エネルギーが大きく高速で走っているときは周辺電子と作用する時間が短く電離量は小さいが，エネルギーを失い止まる寸前になると作用する時間が長くなり電離量は急速に増加する．エネルギーをすべて失い止まってしまうと先の物質とは一切相互作用しない．陽子線やさらにエネルギーの強い炭素線が体内の一定深度で高線量域(Bragg-peak)(図1)を形成し，線量が表面で少なく体内深部で大きくなる理由である．

2．HCCに対する陽子線治療の有効性

1983年11月から1998年7月までの期間，高エネルギー物理学研究機構(KEK)内の陽子線医学研究利用センター(PMRC)において，165例の肝癌患者が第I相/第II相臨床研究としての陽子線治療を施行された．

患側1門または2～3門の固定照射により，線量分布調整体を用い病巣のみをその形状にあわせ選択的に照射された．照射線量の中央値は72Gy(50～88Gy)で平均総線量は72Gy，1回線量の中央値は4.5Gy(2.9～6 Gy)，1回照射平均4 Gy，平均16回照射であった．

成績は図2に示すように，観察期間中の局所

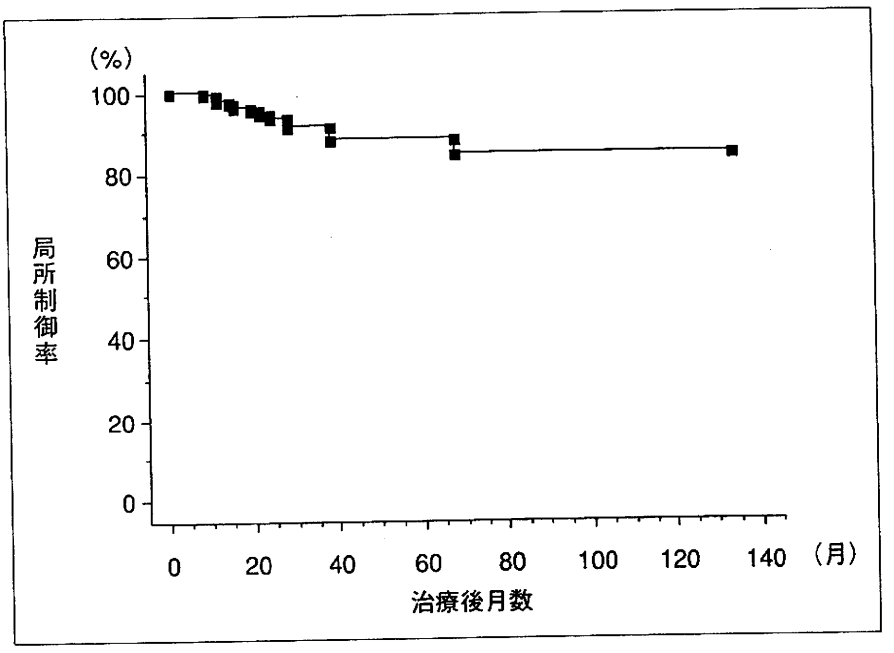

図2　累積局所制御曲線

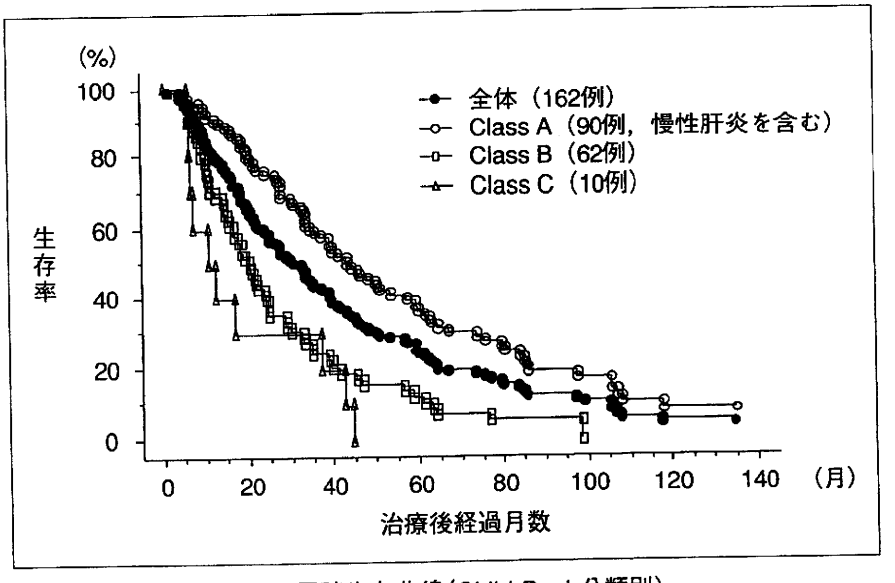

図3　累積生存曲線(Child-Pugh分類別)

制御率は5年局所制御率で88.4%であった[12]．これらに関しては，他施設においてもおおむね同様の成績である．米国においては，本邦よりも成績が悪い[26]．解離の原因は，本邦においては，正確に照射するためにイリジウム針をマーカーとして，腫瘍の辺縁におく．米国はそれを施行せず照射しているため，正確性が劣ると考えられる．微妙な照射方法は重要と考える．全体の生存率は図3のように，1年79.6%(N=129)，2年57.4%(N=93)および5年24.2%(N=35)であった．

表1に示すように多変量解析により，最適な治療効果を得るには，併存肝疾患の重症度と腫瘍数が生存に寄与する因子と判明した．単発で腫瘍径に関係なく，肝機能が慢性肝炎あるいはChild A肝硬変合併肝細胞癌の5年生存率は，52.3%と良好であった．

治療により副作用は，急性期から亜急性期のものは重篤なものはなく，照射終了後には改善するものであった．晩期副反応はbiloma，消化管出血などがあり，胆管や，消化管への影響も考慮しなければならない問題であるかもしれな

表1 陽子線治療後の生存に寄与する因子の解析(Cox比例ハザードモデル)

因子	相対危険率	95%信頼区間	P値
Child-Pughスコア			
Class A*	1.00		
Class B	0.48	1.452-3.032	<0.0001
Class C	0.27	1.879-7.270	0.0002
腫瘍数			
単発	1.00		
多発	0.59	1.165-2.479	0.0059
最大腫瘍径(mm)			
50未満	1.00		
50以上	1.03	0.680-1.391	0.8785
前治療(半年以内)			
あり	1.00		
なし	1.01	0.687-1.428	0.9586

*慢性肝炎，正常肝を含む

い．治療上の大きな利点は有害事象が少なく，治療は痛みを伴わず，治療中の良好なperformance status(PS)を保てることである．

陽子線照射療法は，門脈塞栓例，inferior vena cava(IVC)内腫瘍塞栓例に関してもすべてではないが治療可能であった[11)12)]．患者のQOLを損なうことなく，高度進行肝硬変合併HCCの治療に対しても治療可能となると考えられた．しかし，門脈塞栓例について，どの程度の塞栓まで照射可能であり，良好な効果が得られるかについて，今後evidence-based medicine(EBM)に基づき詳細に検討する必要がある．

3．陽子線照射の適応選択と限界

Digestive Disease Week-Japan(DDW-J)2003における，コンセンサスミーティングにおいて，HCC治療に関して，3 cm以下の場合は現状ではRFAを推奨する，動脈血流の乏しいHCCに対してtranscatheter arterial chemoembolization(TACE)は行うべきでない，2 cm以上の単発で切除可能と判断された場合は，外科的治療が望ましい，とされている[27)]．

陽子線治療の適応はどのようなものであろうか．上述のように，単発で肝予備能が良好な陽子線治療症例における5年生存率は52.3%であり，手術とほぼ同等である．とりわけ，陽子線照射療法において腫瘍の大きさが局所制御や生存率に影響しない点を考慮すれば，焼灼療法の適応になりがたい大きさの単発腫瘍に関しては，肝切除に匹敵しうる治療法となりうる可能性がある．また，尾状葉などの深いところでも十分に照射可能である．現行の治療法で有効とされる3 cm以下の腫瘍に陽子線治療を積極的に行う必要があるか否かは議論されるところである．

PVTTを有する症例においても良好な5年生存率が得られている．さらなる検討が必要であるが，陽子線照射療法は高度進行肝細胞癌においても有用な治療法であることが示唆される．治療上の大きな利点は有害事象が少なく，治療は痛みを伴わず，治療中の良好なPSを保てることである．

肝細胞癌に対する陽子線照射の基本的な適応基準は，現段階で安全に照射できると考えている基準は次のような症例である．(1)手術不適応例でかつ，以下のようなさまざまな理由により局所療法とくにRFAなどの焼灼療法の施行が困難な症例，①3 cmを超える肝細胞癌症例，②Ultrasonography(US)にて描出困難な腫瘍，③肝表面，深部，大血管近傍などの局在により腫瘍への穿刺が困難な場合，(2)肝硬変を含む合併症により既存治療が施行しがたい症例，(3)高齢で既存の局所療法が困難な例，(4)限局的なPVTT, 静脈内塞栓例，などを現段階での適応と考えている．とりわけ多変量解析の結果より，Child-Pugh A肝硬変で単発腫瘍であることが最良の適応であると考えている．表2に現在考えている陽子線治療の位置づけを示す．

陽子線治療はあくまで局所療法であることから，肝細胞癌の臨床的特徴を考慮すると，技術

表2　肝細胞癌に対する陽子線照射適応基準と禁忌

適応：
1. 手術不適例
2. 以下のようなさまざまな理由によりRFAなどの焼灼療法，TACEの施行が困難な肝細胞癌症例
   1) 3 cmを超える肝細胞癌症例
   2) USにて描出困難な腫瘍
   3) 肝表面，深部，大血管近傍などの局在により腫瘍への穿刺が困難な場合
   4) Lipiodol®の集積不良の多血性肝細胞癌
   5) 乏血性腫瘍だが高分化型肝細胞癌を疑う腫瘍
3. 肝硬変を含む合併症により既存治療が施行しがたい症例
4. 限局的なPVTT，静脈内塞栓例，などを現段階での適応としている．

禁忌：
1. 肝内に散在する4個以上の肝細胞癌
2. 総ビリルビン値3.0mg/dl以上
3. 難治性腹水
4. 消化管に近接した腫瘍

PVTT：portal vein tumor thrombosis, RFA：radiofrequency ablation, TACE：transarterial chemoembolization, US：ultrasonography

的には可能であっても，肝内に散在する4個以上の肝細胞癌には適応しがたいと考えている．最後に，総ビリルビン値3.0mg/dl以上，難治性腹水や消化管に近接した腫瘍は，技術的な観点から禁忌であると考えている．また，照射の適応を決めるとき，重要なことは腫瘍と消化管との位置関係である．腫瘍と消化管が最低2 cmは両者が離れていないと，消化管に障害を起こす．難治性の潰瘍や出血を起こすことがある．胃，十二指腸，大腸との位置関係が重要となってくる．また，肝門部の照射も注意が必要である．腸管と胆管の問題がつきまとうことが今後考慮しなければならない課題である．これらは相対的禁忌の範疇であると考えている．

4．重荷電粒子線治療の現状と今後

2006年10月現在で，陽子線治療が施行されているところは，世界で18施設，本邦で6施設である．炭素線は，世界で3施設，本邦で2施設稼働しているのが現状である．全世界で陽子線治療では，42,766名，重イオンで4,520名の患者が治療を受けている．この中でもHCCに対する治療は圧倒的に本邦で施行されている．

HCCに対する重荷電粒子線治療として現在，HCCに対する陽子線治療症例数と，観察期間の長さにおける臨床治験の蓄積は筑波大学の施設が最大規模である[12]．これに次いで，本邦においても，国立がんセンター東病院[13]，米国ロマリンダ大学も報告をしている[26]．陽子線は，最も実用に近い新しい放射線治療というのが大方の一致した見解である．本邦においては筑波大学において，2001年より病院隣接の専用加速器で治療開始されている．国立がんセンター東病院，福井県の敦賀にも若狭湾エネルギー研究センターに陽子線専用施設，兵庫県の播磨公園科学都市に兵庫県立粒子線医療センター，静岡県立静岡がんセンターなどで陽子線治療専用施設が完成しすでに稼働している．また，重イオン治療に関しての臨床試験は1995年より放射線医学総合研究所重粒子医科学センター病院において，Cイオンを用い，HCCに対してphase II臨床成績がすでに報告された[14]．これらもすべてphase I/II試験である．経過観察年数が短いが，陽子線と同等の良好な成果が報告されている．現時点においてはおおむね，陽子線の成績と局所制御に関しては同じである．まだ長期の成果を検討しなければならない．照射線量，照射回数の違いなど，陽子線と手法が若干異なる面があるので，今後陽子線との住み分けをしていく必要があろう．最も新しい施設としては，福島市に民間病院としてはじめて南東北がん陽子線治療センターが2008年10月より稼働している．

これらの施設のほか，重粒子線では，群馬大学が2009年度の稼働を目指して建設中のほか，愛知県大府市が名古屋先進量子医療研究所を設

立し，2010年の稼動を目標にしている．さらに，福井，大阪，神奈川，鹿児島などでも建設計画がある．陽子線では，愛知県名古屋市が西部医療センターに隣接した施設を2010年めどに建設する予定となっている．

これら粒子線治療は現在まだ保険適応になっていないため，治験終了施設においては，250～300万円前後が全額自己負担となっているのが現状である．重荷電粒子線治療の中でも陽子線照射療法は設備などのコストパフォーマンスの面からも進歩，改良がみられつつある．専用加速器の小型化などにより，コストのかかる重イオン治療に比べ，腫瘍を取り扱う専門病院での深部臓器癌治療も将来可能になることは十分に予想されるところである．しかし一方，重粒子線に関しても，小型化，低価格化の検討がなされだしている．今後，重荷電子療法の位置づけをどのようにするかこれも，重要な課題である．

ま と め

肝細胞癌(hepatocellular carcinoma：HCC)に対する全身化学療法に関しては今後，有効性を明確にして，位置づけを確立していかねばならない．一方，放射線治療は従来の照射方法よりも，陽子線，重粒子線は，効果的かつ安全で耐用性に富み，さらには繰り返し可能であることが第II相試験で示された．外科手術同様，根治目的の治療選択肢の一つとして用いられる可能性があり，また腫瘍径や局在，血流，門脈塞栓，合併症などの条件に制限が少なく，HCCに対して幅広い適応を有すると考えられる．しかし，コストや保険診療，randomized controlled trial (RCT)による科学的根拠に基づく有効性の確立など，いくつかの課題も抱えており，今後明確にする必要があろう．

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Original Article

Serum concentration of 27-hydroxycholesterol predicts the effects of high-cholesterol diet on plasma LDL cholesterol level

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Aim: The effect of dietary cholesterol on plasma cholesterol concentrations varies widely among individuals. Recent studies suggest that the synthesis of oxysterols is up-regulated when tissue cholesterol is saturated. The present study was undertaken to test the hypothesis that a serum high concentration of 27-hydroxycholesterol, one of the oxysterols, reflects positive cholesterol balance in the body and predicts intolerance to a high-cholesterol diet.

Methods: In 30 subjects, 750 mg/day of cholesterol was added for 4 weeks to the ordinary diet. Blood samples were collected at the start and finish of the supplementation. Serum sterol and oxysterol concentrations were measured by high-resolution GC-MS.

Results: A receiver operating characteristic curve was drawn and the cutoff point (80 ng/mg cholesterol) was chosen to maximize sensitivity (81.3%) and specificity (64.3%) for predicting a positive change of LDL cholesterol concentration after cholesterol loading. Subjects with higher serum 27-hydroxycholesterol concentrations (≥ 80 ng/mg cholesterol) showed significantly ($P < 0.05$) high values for the change of LDL cholesterol concentration (+7.4 ± 3.4%, mean ± SEM, $n = 17$) compared with those with lower 27-hydroxycholesterol levels (–5.3 ± 2.7%, $n = 13$).

Conclusions: In subjects with high serum 27-hydroxycholesterol concentrations were unable to adapt to a high-cholesterol diet. The concentration of serum 27-hydroxycholesterol appears to reflect cholesterol saturation in the body and predicts to some extent a responsiveness to dietary cholesterol.

Key words: high-cholesterol diet, 27-hydroxycholesterol, hypercholesterolemia, LDL cholesterol, liver X receptor, oxysterol

INTRODUCTION

IN GENERAL, THE intake of dietary cholesterol is believed to increase plasma LDL cholesterol concentrations. Therefore, diets restricted in cholesterol have been recommended for the prevention and treatment of hypercholesterolemia.[1,2] However, the response of plasma cholesterol to dietary cholesterol varies among the population.[3–8] A group of people considered high responders showed significant increases in plasma LDL cholesterol after cholesterol consumption. In contrast, individuals considered low responders showed stable or even decreased LDL cholesterol in spite of high intakes of dietary cholesterol. These facts suggest that the restriction of dietary cholesterol is effective only in high-responding people. Therefore great efforts have been made to explore the mechanism of the individual variability and to predict the responsiveness in each subject before dietary intervention.

The most popular approach is to investigate apolipoprotein (apo) E phenotypes. Subjects with apoE4 phenotype displayed higher plasma cholesterol levels, increased cholesterol absorption, and lower cholesterol synthesis than people with apoE2 phenotype.[9–11]

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However, the effects of apoE phenotype on the response of plasma cholesterol levels to increased dietary cholesterol are still controversial, that is associated[12–15] or not associated[8,16–19] with the responsiveness.

Another approach is to measure the LDL receptor function. Mistry *et al.* reported a negative correlation between the change in plasma cholesterol concentration and LDL receptor activity in peripheral mononuclear leucocytes.[4] In contrast, Homma *et al.* demonstrated that the capacity of the LDL receptor did not explain the variability in the change of plasma cholesterol concentration induced by cholesterol loading.[8]

The aim of the present study was to discover a new biomarker that predicts responsiveness to a high-cholesterol diet. Recent biochemical studies unveiled the regulation of cholesterol metabolism at the molecular level. Cholesterol biosynthesis is down-regulated by oxysterols, intermediates in bile acid biosynthesis, through the modulation of a transcription factor, sterol regulatory element-binding protein (SREBP).[20] The elimination of sterols from the intestine and the liver are also stimulated by oxysterols through the activation of another transcription factor, liver X receptor α (LXRα).[21] Thus oxysterols appear to be messenger molecules that represent positive cholesterol balance in the body. Our results suggested that the baseline serum concentrations of 27-hydroxycholesterol, one of the most abundant oxysterol in human serum,[22] predicted to some extent a responsiveness to dietary cholesterol.

METHODS

Subjects

THIRTY JAPANESE SUBJECTS (11 males and 19 females; aged 29–84 years; BMI 18–28 kg/m^2) were studied, including healthy volunteers and patients with hypercholesterolemia. Patients with hypertension (> 140/90 mmHg, $n = 14$), well-controlled non-insulin-dependent diabetes mellitus (fasting plasma glucose < 126 mg/dL and hemoglobin A_1C < 7.0%, $n = 3$), stable angina pectoris ($n = 2$), old myocardial infarction ($n = 1$), and old cerebral infarction ($n = 2$) were included. Patients with hypocholesterolemia or familial hyperlipoproteinemia were excluded from this study. Informed consent was obtained from all subjects, and the study procedures were in accordance with the ethical standards of the Helsinki Declaration.

Experimental design

A daily dose of 750 mg of cholesterol was added to the ordinary diet for 4 weeks as freeze-dried egg yolk. The subjects were requested not to change their dietary and drinking habits or their exercise patterns. During this study, all subjects were on a free-living Japanese diet that contains 250–350 mg/day cholesterol as estimated from daily food diaries. Patients who received antihyperlipidemic agents were excluded, and the treatment for complications except for hyperlipidemia was continued unaltered during the study period.

At the start and end of supplemental cholesterol feeding, blood samples were collected in the morning before breakfast after an overnight fasting, and serum was stored at −20°C until analyzed.

Chemicals

Sitosterol and campesterol were purchased from Sigma (MO, USA). Lathosterol and 5α-cholestane were obtained from Steraloids (NH, USA). 27-Hydroxycholesterol, 7α-hydroxy-4-cholesten-3-one, [$^2H_7$]27-hydroxycholesterol and [$^2H_7$]7α-hydroxy-4-cholesten-3-one were prepared as described previously.[23]

Measurement of serum cholesterol concentration

Subfractions of serum lipoproteins were obtained by sequential ultracentrifugation.[24] The concentrations of total cholesterol in serum and the lipoprotein subfractions were measured by a Hitachi autoanalyzer (Hitachi, Japan).

Determination of apoE phenotype

ApoE phenotyping was performed by an isoelectric focusing immunoblotting method by Kataoka *et al.*[25]

Assay of LDL receptor activity in lymphocytes

LDL receptor activity was evaluated by the use of peripheral lymphocytes under the method of Ranganathan *et al.*[26] Briefly, mononuclear cells collected by the Ficoll precipitation method were cultured in lipoprotein-deficient medium for 72 h. Nonadherent mononuclear cells (lymphocytes) were collected and incubated with fluorescent LDL at 37°C for 2 h. Fluorescence of the washed lymphocytes was measured with a FACScan flow cytometer (Becton-Dickinson, NJ, USA). The activities in normolipidemic volunteers were measured with every assay to provide an internal control value (100%).

Determination of serum sterol concentrations

Serum levels of sitosterol, campesterol and lathosterol were measured by gas chromatography-mass spectrom-

Table 1 Correlations between baseline serum sterol concentrations and percent change of LDL cholesterol levels by cholesterol loading ($n = 30$)

Serum marker sterols	Change of LDL cholesterol (%)	
	r_s†	*P*-value
Sitosterol (ng/mg cholesterol)	0.000	1.000
Campesterol (ng/mg cholesterol)	0.092	0.631
Lathosterol (ng/mg cholesterol)	0.138	0.466
27-Hydroxycholesterol (ng/mg cholesterol)	0.321	0.083
7α-hydroxy-4-cholesten-3-one (pg/mg cholesterol)	0.037	0.847

†Nonparametric Spearman's rank-order correlation coefficient.

etry (GC-MS). 5α-Cholestane (2 μg) was added to 50 μl of serum as an internal standard, and alkaline hydrolysis was carried out in 1 mL of 1 N ethanolic KOH at 60°C for 1 h. After an addition of 0.5 mL of distilled water, the sterols were extracted twice with 2 mL of n-hexane, and the extract was evaporated to dryness under nitrogen. The extracted sterols were converted into trimethylsilyl (TMS) ethers with 100 μL of TMSI-H (GL Sciences, Japan) for 15 min at 55°C. GC-MS with selected-ion monitoring was performed with a JMS-SX102 instrument equipped with a data processing XMS-system (JEOL, Japan). The accelerating voltage was 10 kV, the ionization energy was 70 eV, the trap current was 300 μA, and the mass spectral resolution was about 10 000. An Ultra Performance capillary column (25 m x 0.32 mm i.d.) coated with methylsilicone (Agilent Technologies, CA, USA) was used at a flow rate of helium carrier gas of 1.0 mL/min. The column oven was programmed to change from 100°C to 260°C at 30°C/min, after a 1-min delay from the start time. The multiple ion detector was focused on *m/z* 357.3521 for 5α-cholestane and sitosterol, *m/z* 343.3364 for campesterol, and *m/z* 458.3943 for lathosterol.

Serum 27-hydroxycholesterol and 7α-hydroxy-4-cholesten-3-one levels were quantified as described previously.[23]

Statistical analysis

Data are expressed as the mean ± SEM. The statistical significance between the results in the different groups was evaluated by a parametric two-sample t-test and a nonparametric Mann-Whitney test. The change of values after cholesterol supplementation was evaluated by a parametric paired t-test and a nonparametric Wilcoxon signed-ranks test. The correlations were tested by calculating Pearson's correlation coefficient, *r*, or a nonparametric Spearman's rank-order correlation coefficient, r_s. Independence was evaluated by Fisher's exact probability test for a 2 × 2 contingency table and by the χ^2-test for a 3 × 2 contingency table. In all the statistical tests, significance was accepted at the level of $P < 0.05$.

RESULTS

Search for a new biomarker that predicts responsiveness to a high-cholesterol diet

THE CORRELATIONS BETWEEN the serum sterol concentrations and percent change of LDL cholesterol levels by cholesterol loading are summarized in Table 1. Although no statistically significant correlation was observed, a relatively low *P*-value was obtained for the relationship of 27-hydroxycholesterol concentrations with the percent changes of LDL cholesterol.

Figure 1a depicts the relationship and Figure 1b represents a receiver operating characteristic (ROC) curve to determine a cutoff point of the 27-hydroxycholesterol concentration that optimally discriminated the subjects with positive changes of serum LDL cholesterol by cholesterol loading from those with negative changes. The cutoff point was chosen to maximize sensitivity and specificity, and it was 80 ng/mg cholesterol; the sensitivity and specificity for predicting a positive change of LDL cholesterol concentration because of cholesterol loading were 81.3% and 64.3%, respectively.

Characteristics of subjects with high serum 27-hydroxycholesterol concentrations

The baseline characteristics of subjects with low (< 80 ng/mg cholesterol) and high (≥ 80) serum 27-hydroxycholesterol concentrations were compared in Table 2. The subjects with low 27-hydroxycholesterol concentrations were all females, whereas 65% of those with high 27-hydroxycholesterol concentrations were males. The concentrations of HDL cholesterol were significantly low in subjects with high (≥ 80 ng/mg choles-

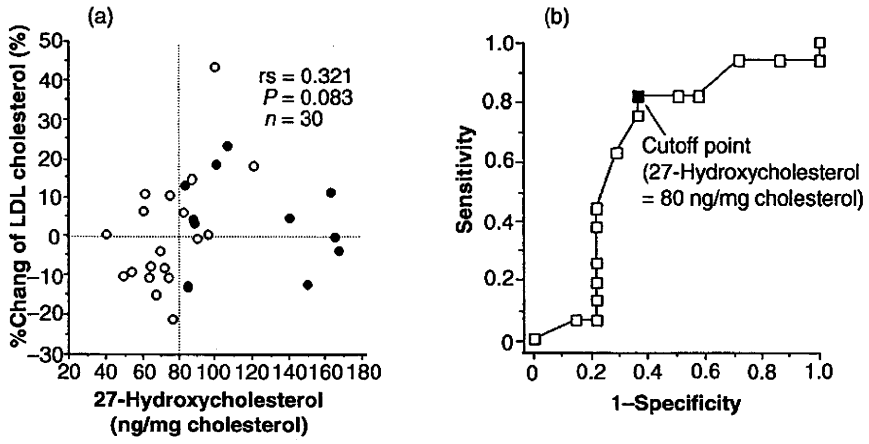

Figure 1 Relationships of baseline serum 27-hydroxycholesterol concentrations with % of change of LDL cholesterol concentrations by cholesterol loading (a), and a receiver operating characteristic (ROC) curve for determining a cutoff point of the 27-hydroxycholesterol concentration that optimally discriminated the subjects with positive changes of serum LDL cholesterol by cholesterol loading from those with negative changes (b). The percent change of LDL cholesterol concentration was calculated as (concentration after cholesterol loading – concentration before loading)/concentration before loading x100%. The open circles indicate female subjects ($n = 19$), and the closed circles represent male subjects ($n = 11$).

terol) serum 27-hydroxycholesterol concentrations. The other baseline data, including LDL receptor activity and apoE phenotype, were not significantly different between the two groups.

Figure 2 compares individual responses of serum lipid concentrations after cholesterol loading between the subjects with low (< 80 ng/mg cholesterol) and high (≥ 80) baseline serum 27-hydroxycholesterol concentrations. The percent change of LDL cholesterol was significantly higher in subjects with high baseline 27-hydroxycholesterol concentrations than in those with low concentrations (Fig. 2b). The percent change of total cholesterol also tended to be high in subjects with high baseline 27-hydroxycholesterol concentra-

Table 2 Baseline characteristics of subjects with high serum 27-hydroxycholesterol versus low serum 27-hydroxycholesterol concentrations

	27-Hydroxycholesterol concentration (ng/mg cholesterol)		
	Low† (< 80)	High† (≥ 80)	*P*-value‡
n (male/female)	0/13	11/6	< 0.0005
Age (years)	63.6 ± 4.5§	62.5 ± 2.7	0.63
BMI (kg/m^2)	22.3 ± 0.7	23.1 ± 0.6	0.43
Total cholesterol (mg/dL)	246 ± 14	229 ± 11	0.34
LDL cholesterol (mg/dL)	164 ± 13	143 ± 9	0.16
HDL cholesterol (mg/dl)	66 ± 4	52 ± 4	< 0.05
LDL receptor activity (%)¶	112 ± 5	106 ± 5	0.36
ApoE phenotype (E2/E3/E4)††	1/8/4	0/13/4	0.43

†Each subject was assigned to one of the two groups by serum 27-hydroxycholesterol concentration; Low, < 80 ng/mg cholesterol; High, ≥ 80 ng/mg cholesterol.
‡The *P*-value for gender was calculated by Fisher's exact probability test and that for apoE phenotype by the χ^2-test for independence. The other *P*-values were calculated by the nonparametric Mann–Whitney test. §All such values are mean ± SEM.
¶The activities in normolipidemic volunteers were measured with every assay to provide an internal control value (100%).
††E2, E2/2 + E3/2; E3, E3/3; E4, E4/3 + E4/4.

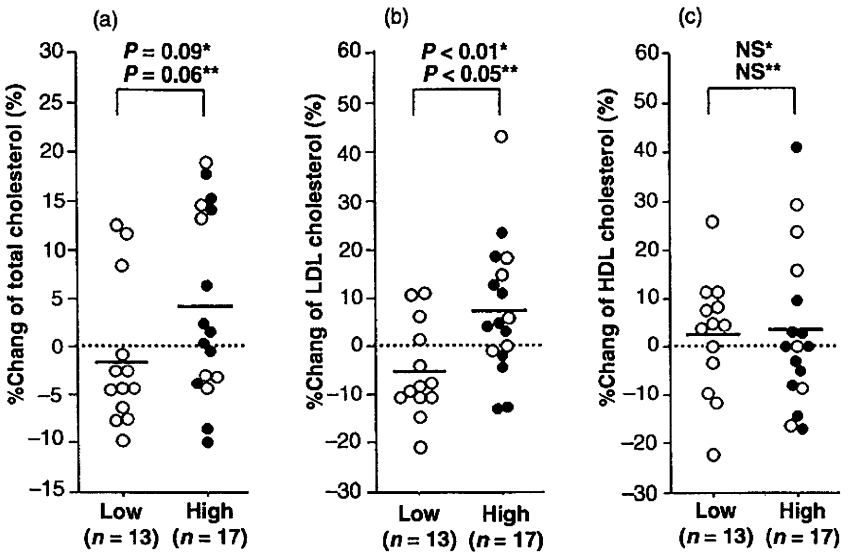

Figure 2 Comparison of the individual responses of serum total cholesterol (a), LDL cholesterol (b), and HDL cholesterol (c) concentrations after cholesterol loading between the subjects with low baseline serum 27-hydroxycholesterol levels (< 80 ng/mg cholesterol) and those with high levels (≥ 80). The percent changes of these plasma sterol concentrations were calculated as (concentration after cholesterol loading – concentration before loading)/concentration before loading x100%. The mean value for each group is indicated by a horizontal line. The open circles indicate female subjects ($n = 19$), and the closed circles represent male subjects ($n = 11$). *Analyzed by a parametric two-sample t-test. **Analyzed by a nonparametric Mann–Whitney test.

tions, but the difference was not statistically significant (Fig. 2a). In contrast, the percent change of HDL cholesterol was not significantly different between subjects with low baseline 27-hydroxycholesterol concentrations and those with high concentrations (Fig. 2c).

Effects of cholesterol loading on serum 27-hydroxycholesterol concentrations

As shown in Figure 3, strong positive correlations were observed between baseline 27-hydroxycholesterol concentrations and the concentrations after cholesterol loading ($r = 0.851$, $P < 0.0001$; $r_s = 0.911$, $P < 0.0001$). Furthermore, serum 27-hydroxycholesterol concentrations before and after cholesterol loading were compared by a parametric paired t-test and a nonparametric Wilcoxon signed-ranks test (92.2 ± 6.4 vs. 88.2 ± 5.2 ng/mg cholesterol), and no significant change was observed. Therefore similar results were obtained even if 27-hydroxycholesterol concentrations after cholesterol loading were used as a predictor instead of baseline 27-hydroxycholesterol concentrations. When the same 80 ng/mg cholesterol was used as a cut-off value for 27-hydroxycholesterol concentration after cholesterol loading, the percent change of LDL cholesterol was significantly higher in subjects with high

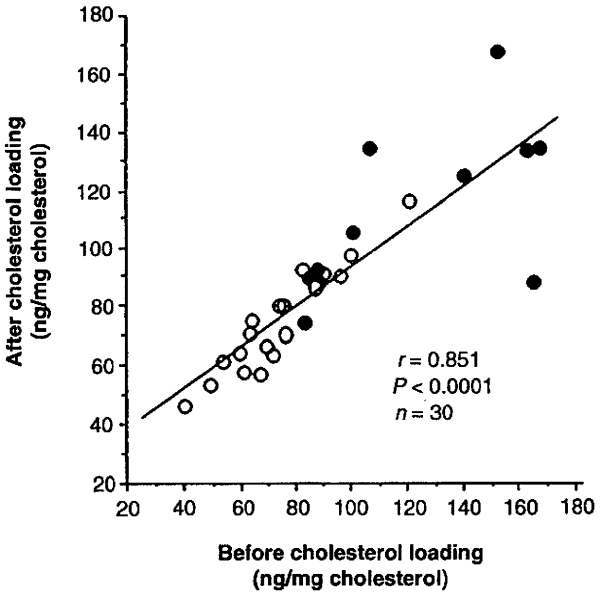

Figure 3 Relationships of serum 27-hydroxycholesterol concentrations before cholesterol loading (baseline concentrations) with those after cholesterol loading. The open circles indicate female subjects ($n = 19$), and the closed circles represent male subjects ($n = 11$).

(≥ 80) 27-hydroxycholesterol concentrations than in those with low (< 80) concentrations [+7.1 ± 3.6% (n = 16) vs. −4.0 ± 2.6% (n = 14); $P < 0.05$, significantly different by both the two-sample Student t-test and the Mann–Whitney test].

DISCUSSION

SERUM CONCENTRATIONS OF several marker sterols reflect cholesterol metabolism in the body. First, serum concentrations (relative to cholesterol) of plant sterols, sitosterol and campesterol, are positively correlated with the fractional absorption of dietary cholesterol and negatively correlated with fecal endogenous cholesterol outputs.[27,28] Second, serum concentration (relative to cholesterol) of lathosterol, a cholesterol precursor, reflects whole body cholesterol synthesis[29] or hepatic activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the cholesterol biosynthesis.[30] Third, serum concentration (relative to cholesterol) of 7α-hydroxy-4-cholesten-3-one, an intermediate in bile acid synthesis, has been used as a marker for hepatic activity of cholesterol 7α-hydroxylase (CYP7A1),[23] the rate-limiting enzyme in the classic bile acid biosynthetic pathway, and total bile acid synthesis.[31] To explore a biomarker that might predict responsiveness to cholesterol intake, we tried to measure serum baseline concentrations of the above sterols. However, they were not at all correlated with the percent change of LDL cholesterol by cholesterol loading (r_s = 0.000–0.138, P = 1.000–0.466).

Serum 27-hydroxycholesterol is another candidate for a predictor of cholesterol responsiveness. In fact, although the result did not reach statistical significance, a higher correlation coefficient was obtained between baseline 27-hydroxycholesterol concentrations and the percent change of LDL cholesterol because of cholesterol loading (r_s = 0.321, P = 0.083). This sterol is synthesized by CYP27A1 that is expressed in many tissues, including liver,[32] intestine,[32] vascular endothelium,[33] macrophages,[34] and atherosclerotic plaque.[35] CYP27A1 seems to protect the human body from cholesterol overload by at least three concurrent but separate mechanisms. The first mechanism operates via a suppression of cholesterol biosynthesis[36] by the inhibition of SREBP2 processing.[37] The second, 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid (immediate metabolite of 27-hydroxycholesterol by the same CYP27A1), are more polar than cholesterol and are transported into the liver and metabolized to bile acids more easily than cholesterol.[38] The third, 27-hydroxycholesterol, is one of the endogenous ligands for LXRα and inhibits the accumulation of cholesterol by activating this nuclear receptor.[39]

Cholesterol homeostasis in mammals is maintained by a balance between absorption from the intestine, *de novo* synthesis in the liver and extrahepatic tissues, and excretion to the bile as cholesterol or bile acids.[40] Our results that subjects with high baseline serum 27-hydroxycholesterol concentrations (≥ 80 ng/mg cholesterol) showed a higher percent change of LDL cholesterol by cholesterol loading suggest that these subjects had positive cholesterol balance in the body and less extra capacity to preserve serum LDL cholesterol concentrations after cholesterol loading. Thus serum 27-hydroxycholesterol concentrations seemed to predict to some extent the responsiveness to dietary cholesterol.

Since serum 27-hydroxycholesterol concentrations were fairly stable and not significantly affected by cholesterol loading (Fig. 3), the concentration appears to be determined by endogenous factors rather than dietary cholesterol. Therefore it may also be true that a restriction of cholesterol results in a more effective reduction of serum LDL cholesterol in subjects with high serum 27-hydroxycholesterol concentrations compared to those with low concentrations. An interesting finding in the present study is that the subjects with low 27-hydroxycholesterol concentrations (< 80 ng/mg cholesterol) were all females, and 65% of the subjects with high 27-hydroxycholesterol concentrations (≥ 80) were males (Table 2). Several studies have suggested that a low cholesterol diet reduces serum total cholesterol and LDL cholesterol concentrations more greatly in males than in females,[41,42] which may be explained in part by our idea that serum 27-hydroxycholesterol concentrations predict the effects of cholesterol restriction on serum LDL cholesterol.

The importance of serum 27-hydroxycholesterol concentrations and CYP27A1 activity in hepatic and extrahepatic tissues for the response to dietary cholesterol has also been pointed out in experiments using baboons.[43,44] However, the conclusions are completely different from humans. In baboons, baseline serum 27-hydroxycholesterol concentrations were not significantly different between high and low responders, and with a high-cholesterol diet, a significant elevation of 27-hydroxycholesterol concentrations was observed only in the low-responding baboons. Although we excluded subjects with hypocholesterolemia in our study, it may be possible that the treatment of hypocholesterolemic patients with high-cholesterol diets

shows results similar to those of the baboons because basal serum LDL cholesterol concentrations in baboons are very low (less than HDL cholesterol).

In our results, the concentrations of HDL cholesterol were significantly low in subjects with high serum 27-hydroxycholesterol concentrations (Table 2). LXRα upregulates the expression of cholesteryl ester transfer protein (CETP) and CETP transfers cholesteryl ester from HDL to other lipoproteins, so that serum HDL cholesterol levels are reduced. Thus high serum 27-hydroxycholesterol concentrations may reflect the activation of LXRα *in vivo*. A recent report by Higuchi *et al.*[45] suggests that the activation of LXRα is one of the important factors that cause nonalcoholic fatty liver disease (NAFLD) in humans. Further investigations are expected to use serum oxysterol markers for the evaluation of hepatic LXRα activity.

In summary, serum high 27-hydroxycholesterol concentrations were thought to reflect positive cholesterol balance in the body and predict, to some extent, a responsiveness to dietary cholesterol loading. A determination of serum 27-hydroxycholesterol concentrations seems to be useful in predicting tolerance to a high-cholesterol diet and the effects of cholesterol restriction therapies.

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methods

Highly sensitive quantification of key regulatory oxysterols in biological samples by LC-ESI-MS/MS[S]

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Abstract We describe a highly sensitive and specific method for the quantification of key regulatory oxysterols in biological samples. This method is based upon a stable isotope dilution technique by liquid chromatography-tandem mass spectrometry (LC-MS/MS). After alkaline hydrolysis of human serum (5 μl) or rat liver microsomes (1 mg protein), oxysterols were extracted, derivatized into picolinyl esters, and analyzed by LC-MS/MS using the electrospray ionization mode. The detection limits of the picolinyl esters of 4β-hydroxycholesterol, 7α-hydroxycholesterol, 22R-hydroxycholesterol, 24S-hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol, and 24S,25-epoxycholesterol were 2–10 fg (5–25 amol) on-column (signal-to-noise ratio = 3). Reproducibilities and recoveries of these oxysterols were validated according to one-way layout and polynomial equation, respectively. The variances between sample preparations and between measurements by this method were calculated to be 1.8% to 12.7% and 2.9% to 11.9%, respectively. The recovery experiments were performed using rat liver microsomes spiked with 0.05 ng to 12 ng of oxysterols, and recoveries of the oxysterols ranged from 86.7% to 107.3%, with a mean recovery of 100.6%. This method provides reproducible and reliable results for the quantification of oxysterols in small amounts of biological samples.— Honda, A., K. Yamashita, T. Hara, T. Ikegami, T. Miyazaki, M. Shirai, G. Xu, M. Numazawa, and Y. Matsuzaki. **Highly sensitive quantification of key regulatory oxysterols in biological samples by LC-ESI-MS/MS. *J. Lipid Res.* 2009. 50: 350–357.**

Supplementary key words liquid chromatography-tandem mass spectrometry • electrospray ionization • 24S,25-epoxycholesterol • 4β-hydroxycholesterol • 7α-hydroxycholesterol • 22R-hydroxycholesterol • 24S-hydroxycholesterol • 25-hydroxycholesterol • 27-hydroxycholesterol

Biological samples contain a large number of oxysterols (1), and most of them are formed from cholesterol by enzymatic oxidation (2–6) (**Fig. 1**) or autoxidation (7). By contrast, the oxysterol 24S,25-epoxycholesterol is not derived from cholesterol but is produced de novo from acetyl-CoA via a shunt in the mevalonate pathway (8).

These oxysterols are important molecules for preserving lipid homeostasis in the body. 7α-Hydroxycholesterol is a product of CYP7A1, which is the rate-limiting enzyme in the classic bile acid biosynthetic pathway. 27-Hydroxycholesterol, 24S-hydroxycholesterol, 4β-hydroxycholesterol, 22R-hydroxycholesterol, and 24S,25-epoxycholesterol are effective endogenous ligands of the nuclear receptors liver X receptor α (LXRα) and LXRβ (9–11). In addition, 27-hydroxycholesterol (12), 25-hydroxycholesterol (13), and 24S,25-epoxycholesterol (14) are known to downregulate the cholesterol biosynthetic pathway, presumably by blocking the processing of the sterol-regulatory element binding protein.

GC-MS has historically been used for the analyses of oxysterols in serum and tissues (1, 15) because the sensitivity and specificity of conventional GC with flame ionization detector is not sufficient to quantify oxysterols in biological samples. However, GC-MS is still not an ideal method, especially for the analysis of 24S,25-epoxycholesterol, because this epoxycholesterol does not survive the temperature required for GC analysis (16). Another approach to quantifying oxysterols in biological samples was HPLC with ultraviolet (UV) detection after derivatization to the Δ^4-3-ketones (16–19). This method made it possible to detect

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Abbreviations: CTX, cerebrotendinous xanthomathosis; ESI, electrospray ionization; LC-APCI-MS, liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LXRα, liver X receptor α; SRM, selected reaction monitoring; TMS, trimethylsilyl.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of three tables.

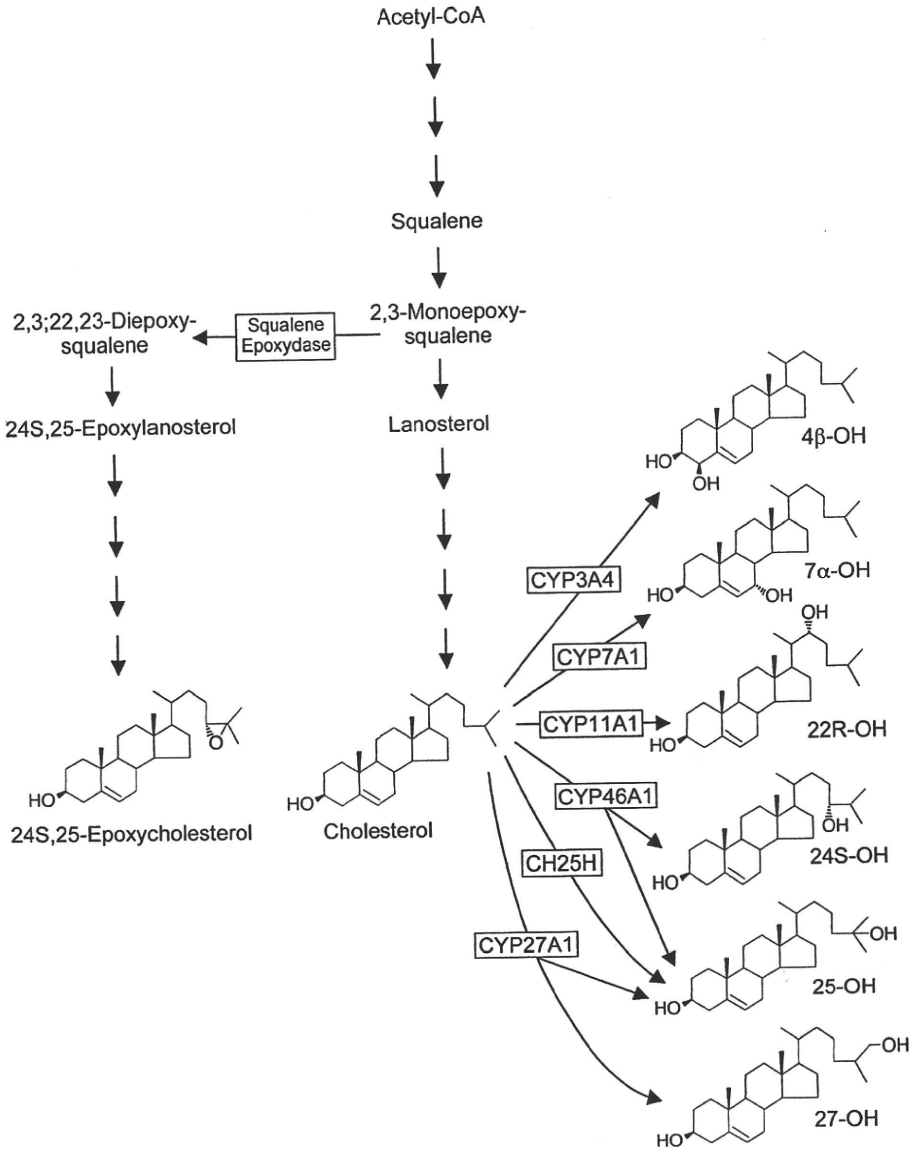

Fig. 1. Biosynthetic pathways for key regulatory oxysterols. Hydroxycholesterols are synthesized from cholesterol, whereas 24S,25-epoxycholesterol is derived from a shunt in the cholesterol biosynthetic pathway. CH25H, cholesterol 25-hydroxylase; 4β-OH, 4β-hydroxycholesterol; 7α-OH, 7α-hydroxycholesterol; 22R-OH, 22R-hydroxycholesterol; 24S-OH, 24S-hydroxycholesterol; 25-OH, 25-hydroxycholesterol; and 27-OH, 27-hydroxycholesterol.

the 24S,25-epoxycholesterol derivative as an intact form, but the lower limit of detection for the Δ^4-3-ketones of oxysterols was about 2 ng on-column (16), which was not sufficient for quantification of the oxysterols in a small amount of biological sample.

Recently, liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS) was introduced as a sensitive, specific, and rapid method for the quantification of oxysterols (20, 21). In addition, LC-tandem mass spectrometry (LC-MS/MS) using electrospray ionization (ESI) has also been applied to the analysis of oxysterols (22). In general, ESI is not the best ionization method for neutral steroids because of its poor ionization efficiency. However, our recent study demonstrated that the derivatization of monohydroxysterols into picolinyl esters markedly enhanced the ionization efficiency in the ESI process, and the method was much more sensit than the assay of native monohydroxysterols by LC-AP MS/MS (23). In this study, we have applied our derivat tion method to dihydroxy- and epoxysterols. In each c singly charged ions were observed as the base peaks in positive ESI mass spectra and amol levels of these oxyste were detectable.

MATERIALS AND METHODS

Chemicals

4β-Hydroxycholesterol (cholest-5-en-3β,4β-diol), 7α-hydr cholesterol (cholest-5-en-3β,7α-diol), 22R-hydroxycholest (cholest-5-en-3β,22R-diol), 24S-hydroxycholesterol (cholest-5 3β,24S-diol), 25-hydroxycholesterol (cholest-5-en-3β,25-d