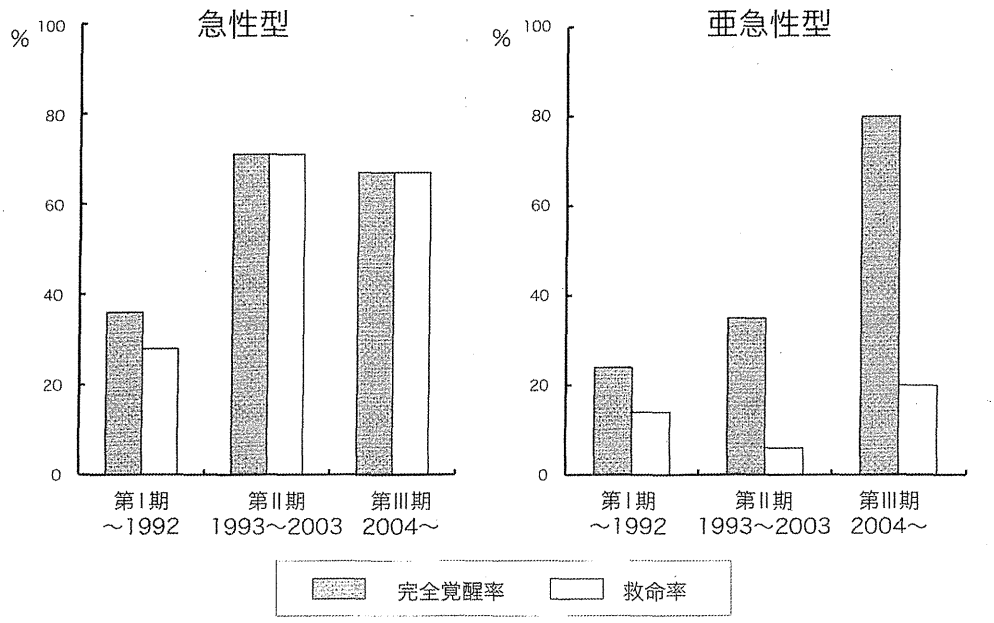

図2　人工肝補助の手法の変遷と昏睡覚醒率および救命率との関連(岩手医大消化器・肝臓内科)
第Ⅰ期：血漿交換のみ，第Ⅱ期：血漿交換＋持続血液濾過透析，
第Ⅲ期：血漿交換＋高流量血液濾過透析＋持続血液濾過透析

アミノフェン以外の急性肝不全に対して用いることは一般的ではないが，急性重症肝障害の昏睡発現予防治療薬の候補として検討が進められている．

3. 人工肝補助療法

欧米では，MARS[28]，血液透析[29]，血漿交換[30]などの比較試験を行っているが，いずれも救命率の改善を証明できず，普及に至っていない．また，欧米ではブタ肝細胞(HepatAsist)あるいは肝芽腫細胞(ELAD)などを用いた生物学的人工肝臓の開発も進められている．一部の報告で急性肝不全の救命効果が示されている[31]が，肝移植も含めての成績であり，単独療法での明確な効果はいまだに示されていない．

これに対して，わが国では，人工肝補助療法が劇症肝炎に対する内科治療の主体である．血漿交換単独の時代に比較し，持続濾過透析(HDF)の導入により，少なくとも専門施設における昏睡覚醒率および急性型の救命率はめざましく向上した印象がある(図2)．しかし，各施設の症例数に限りがあり，しかも，施設ごとの手法に差があるため，比較試験が行われず，エビデンスとして確立していないのが現状である．

この状況に対し，厚生労働省科学研究費補助金(難治性疾患克服事業)「難治性の肝・胆道疾患に関する調査研究」班劇症肝炎分科会では，2011年，血液浄化法の有効性評価を目的としたワーキンググループ(WG)を発足し，オンラインHDFを中心とした人工肝補助の有効性の検討を開始した．このWGの研究により，わが国の人工肝補助の客観的評価が確立されると期待される．

4. 合併症予防治療

わが国における劇症肝炎の合併症の頻度を表7に示す．急性肝不全は細菌および真菌感染の危険があり[32～34]，これらが全身性炎症

表7　わが国における劇症肝炎の合併症とその頻度(2004～2009，文献11より改変)

	全症例 (n=488)	劇症肝炎急性型 (n=277)	劇症肝炎亜急性型 (n=233)	遅発性肝不全 (n=28)
感染	35.8	32.9	36.7	51.9
脳浮腫	18.7	24.1	13.0	22.7
消化管出血	13.6	11.0	15.4	20.0
腎不全	38.9	40.9	37.0	39.3
播種性血管内血液凝固	35.7	35.7	33.6	53.8
心不全	7.5	8.9	5.6	12.0

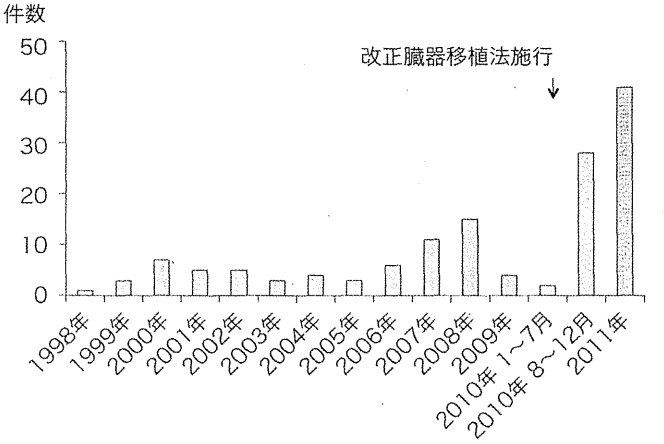

図3　わが国における脳死肝移植件数の変遷
(日本臓器移植ネットワークホームページ)

症候群(SIRS)を引き起こし，ひいては脳症，脳浮腫の進展を助長する[35,36]．予防的な抗生剤，抗真菌薬の投与が感染症を予防することは示されているが，救命率向上の効果は明らかではない[35,36]．

急性肝不全に限らず，ICU管理あるいは人工呼吸管理の患者では消化管出血が多く，ヒスタミン2受容体(H2)阻害薬や胃粘膜保護剤の予防投与の有効性が示されている[37,38]．急性肝不全においてもRCTではないが，H2阻害薬の有効性が示されている[39]．

脳浮腫は，III度以上の肝性脳症に合併する頻度が高く，不可逆な脳障害を引き起こすため，肝移植の適応禁忌になっている．発症機序[40,41]から，感染あるいはSIRSの予防，体液管理，高アンモニア血症対策が重要といわれる．先にも述べたように，わが国で行われている血液濾過透析を主体とした体液管理は，これらを包含した効果があり，脳浮腫予防のみならず少なくとも急性型の救命率向上に大きく寄与したと考えられる(図2)が，比較試験が行われていないためエビデンスになっていない．

5. 肝移植

わが国では劇症肝炎の約25％に肝移植が行われており，そのほとんどは生体肝移植である．1年，5年，10年生存率はそれぞれ75％，70％，69％と報告されており，内科治療の成績より遙かに高い[42]．しかも，肝移植は原則として内科的治療による救命を断念し

表8　急性肝不全の予後予測スコア：肝移植適応判定への応用（文献43より改変）

ポイント	0	1	2
発症－昏睡期間（日）	≦5	6～10	11≦
プロトロンビン時間（%）	20<	5<≦20	5.0≦
総ビリルビン（mg/dl）	<10	10～15	15≦
直接/間接ビリルビン比	0.7≦	0.5≦<0.0.7	<0.5
血小板（万/μl）	10<	5<≦10.0	≦5.0
肝萎縮	なし	あり	

脳症発現時のデータより評価

総得点	死亡割合（%）
9≦	90.0
8	96.3
7	91.3
6	85.5
5	73.8
4	56.3
3	24.0
2	20.0
1	8.0
0	0.0

た患者に対して行われることから，その意義は極めて大きい．

肝移植の適応に関しては，2011年より厚生労働省の班会議で作成された移植適応スコア[43]が推奨されている（表8）．この基準ではスコアにより内科治療の救命割合が提示されているのが特徴である．昏睡発現時のスコアが5点以上が移植適応とされるが，スコア4点でも約半数が死亡することから，先に述べた肝移植による救命率と比較して，本人および家族の意向により治療法を選択する目安となる（表8）．

わが国における脳死肝移植は，1997年に脳死臓器移植法が成立し，1998年に第1例目が行われたが，2009年までの10年間で66例が行われたにすぎなかった．そのうち，劇症肝炎は8例（14％）である．しかし，2010年7月に改正臓器移植法が施行されてからは，17カ月で69例の移植が行われている（図3）．劇症肝炎は，レシピエントの医学的緊急度が最上位にあることから，ドナーの増加により劇症肝炎に対する脳死肝移植が増加することが期待される．

欧米においても，急性肝不全における肝移植の救命効果についてはRCTがなされていない．しかし，無作為ではないが移植例と内科治療例との救命率の比較から，肝移植が急性肝不全の救命率を有意に向上すると考えられている[44]．米国での急性肝不全に対する脳死肝移植の1年生存率は近年82％に向上したといわれる[45]．英国では1年，5年生存率はそれぞれ81％，73％と報告されている[46]．日本と異なり，欧米では脳死肝移植がほとんどを占め，米国では急性肝不全に対する生体肝移植は，移植全体のわずか2％である．

移植適応の判定に関しては，King's CollegeやClichyはじめ種々の予後予測式や新しい生化学マーカーが報告されているが，いずれ

も満足すべきものではない[10].

6 おわりに

わが国では，急性肝不全(劇症肝炎)に関する集計は綿密に行われているが，治療介入に関するエビデンスに乏しいのが現状である．人工肝補助技術をはじめとしたわが国の優れた治療技術がエビデンスとして世界に発信されることを期待したい．

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特別講演

重症肝炎克服に向けた取り組み

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Key words：*fulminant hepatitis, acute liver failure, prediction of encephalopathy development, artificial liver support, prothrombin time*

I. はじめに

急性肝炎はウイルスや薬物により引き起こされる肝の急性炎症で，薬物の早期中止やウイルス感染の終息により自然回復することが多い．しかし，約 1％が劇症肝炎を発症し，その場合は大半が死の転帰をとる．本稿では，肝炎劇症化の予知・予防，劇症肝炎の人工肝補助療法，新規治療法開発の動向を解説する．

II. 重症肝炎の概念と定義

「劇症肝炎」は日本では重症肝炎の典型として，広く知られた疾患名であるが，世界的には必ずしも普及した概念・疾患名ではない．世界的には一般に「急性肝不全」と言う疾患群名が用いられる．急性肝不全（acute hepatic failure）とは「急激かつ高度の肝細胞機能障害に基づいて肝性昏睡をはじめとする肝不全症状を来す予後不良の疾患群」と定義される[1]．原因は肝炎に限らず，薬物中毒や循環障害，代謝疾患などを含む疾患群（症候群）である．これに対し，劇症肝炎（fulminant hepatitis）は，急性肝不全のうちウイルス性肝炎，薬剤アレルギー性肝炎，自己免疫性肝炎（急性発症）を原因とする，いわゆる肝炎に限定される．日本ではウイルス性が大多数と考えられてきたことから，これまで劇症肝炎が急性肝不全の代表として扱われてきた経緯がある．しかし，その約 1/3 が成因不明であり，これらが果たして肝炎なのかは明確ではない．これらの問題点を解消し，世界共通の概念との整合性を得る目的から，我が国でも急性肝不全の概念が導入され，2011 年厚生労働省科学研究費補助金（難治性疾患克服事業）「難治性の肝・胆道疾患に関する調査研究」班 において診断基準が定められた（表 1）[2]．

各国の定義も同様で，診断基準には「高度の肝機能障害」の客観的指標として，肝の蛋白合成能を表す prothrombin time（PT）を採用している[3]．我が国では PT 40%以下または PT INR 1.5 以上を示す急性肝障害を急性肝不全と定めている．従来の劇症肝炎は，肝炎による昏睡型急性肝不全に相当する[2]．昏睡型の急性肝不全の予後は発症あるいは黄疸の発現から肝性昏睡の発現までの期間により異なることが知られており，この期間によっていくつかの臨床病型に分けられている[4-5]．我が国では劇症肝炎の全国集計を基

* 平成 23 年 11 月 27 日に岩手県盛岡市にて開催された第 127 回岩手医学会総会における特別講演．

表 1. 急性肝不全の定義
（厚生労働省「難治性の肝・胆道疾患に関する調査研究」班，持田，2011）

正常肝ないし肝予備能が正常と考えられる肝に肝障害が生じ，初発症状出現から 8 週以内に，高度の肝機能障害に基づいてプロトロンビン時間が 40% 以下ないしは INR 値 1.5 以上を示すものを「急性肝不全」と診断する．

急性肝不全は肝性脳症が認められない，ないしは昏睡度が I 度までの「非昏睡型」と，昏睡 II 度以上の肝性脳症を呈する「昏睡型」に分類する．

また，「昏睡型急性肝不全」は初発症状出現から昏睡 II 度以上の肝性脳症が出現するまでの期間が 10 日以内の「急性型」と，11 日以降 56 日以内の「亜急性型」に分類する．

（注 1）B 型肝炎ウイルスの無症候性キャリアからの急性増悪例は「急性肝不全」に含める．また，自己免疫性で先行する慢性肝疾患の有無が不明の症例は，肝機能障害を発症する前の肝機能に明らかな低下が認められない場合は「急性肝不全」に含めて扱う．
（注 2）アルコール性肝炎は原則的に慢性肝疾患を基盤として発症する病態であり，「急性肝不全」から除外する．但し，先行する慢性肝疾患が肥満ないしアルコールによる脂肪肝の症例は，肝機能障害の原因がアルコール摂取ではなく，その発症前の肝予備能に明らかな低下が認められない場合は「急性肝不全」として扱う．
（注 3）薬物中毒，循環不全，妊娠脂肪肝，代謝異常など肝臓の炎症を伴わない肝不全も「急性肝不全」に含める．ウイルス性，自己免疫性，薬物アレルギーなど肝臓に炎症を伴う肝不全は「劇症肝炎」として扱う．
（注 4）肝性脳症の昏睡度分類は犬山分類（1972 年）に基づく．但し，小児では「第 5 回小児肝臓ワークショップ（1988 年）による小児肝性昏睡の分類」を用いる．
（注 5）成因分類は「難治性の肝疾患に関する研究班」の指針（2002 年）を改変した新指針に基づく．
（注 6）プロトロンビン時間が 40% 以下ないしは INR 値 1.5 以上で，初発症状出現から 8 週以降 24 週以内に昏睡 II 度以上の脳症を発現する症例は「遅発性肝不全」と診断し，「急性肝不全」の類縁疾患として扱う．

に，初発症状から昏睡までの期間が 10 日以内の急性型と 11 日以上の亜急性型に分類している（表 1）．発症 - 昏睡期間のさらに長い遅発性肝不全は，極めて予後不良で，急性肝不全の類縁疾患とされる．わが国における劇症肝炎の内科的救命割合の変遷と肝移植割合を図 1 に示す．

III. 急性肝不全の発症機序

急性肝不全の基本的な病態は肝細胞機能障害，肝再生不全，肝性脳症である．急激かつ高度の肝細胞機能障害の原因は病理組織学的には広範性あるいは亜広範性の肝細胞死による場合がほとんどで，肉眼的に肝は赤色肝萎縮あるいは黄色肝萎縮を呈する．広範壊死を来した肝は再生不全に陥り，代謝能低下に伴う肝性脳症（代謝性脳症）を発現する．

ウイルス肝炎における肝細胞障害機序として，感染細胞に表出されるウイルス抗原を，細胞障害性 T 細胞が攻撃することにより，肝細胞アポトーシス[6-10]が惹起されると考えられている．通常のウイルス性急性肝炎では，このような機序で巣状の壊死にとどまるが，急性肝不全では広汎肝細胞死にまで至る．この機序には被感染者（宿主）の過剰な炎症・免疫反応[11-15]やこれに伴う循環障害[16-18]が想定されている．一方，ウイルス側の要因として，ウイルス遺伝子の変異による抗原性あるいは増殖力，蛋白転写活性の変化が想定されている[19]．

IV. 肝炎劇症化の予知・予防

昏睡発現後の急性肝不全の内科的救命率は非常に低い（図 1）ことから，昏睡発現を予知し，早期に治療開始することによってこれを阻止あるいは予後改善に繋げる試みがなされている[20-21]．岩手医大消化器肝臓内科を中心専門施設とする，北東北の急性肝障害診ネットワークでは，劇症化予知式に基づいた広域の患者搬送システムを構築しプロスペク

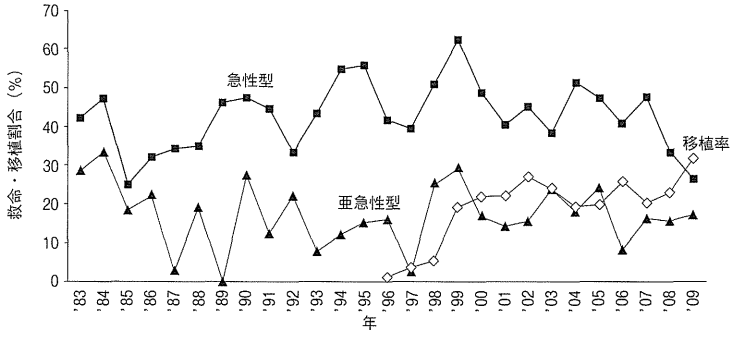

図 1. わが国における劇症肝炎の内科的救命率と肝移植率の変遷
厚生労働省科学研究費補助金（難治性疾患克服事業）「難治性の肝・胆道疾患に関する調査研究」班による全国集計結果より作成

表 2. わが国における劇症肝炎の治療法とその施行率
(2004-2009 厚生労働省全国集計)

	全症例 (n=488)	劇症肝炎急性型 (n=277)	劇症肝炎亜急性型 (n=233)	遅発性肝不全 (n=28)
ステロイド	73.4	68.3	76.4	89.3
グルカゴン－インスリン療法	14.4	13.7	14.7	17.9
特殊組成アミノ酸	20.2	14.3	23.6	39.3
血漿交換	89.8	92.5	89.3	71.4
血液濾過透析	74.0	75.1	74.9	57.1
プロスタグランディン E1	6.8	6.7	7.3	3.6
インターフェロン	13.9	15.4	12.9	10.7
サイクロスポリン A	10.0	7.0	12.9	10.7
核酸アナログ	38.6	50.9	27.5	32.1
抗凝固療法	46.7	43.2	51.1	39.3
肝移植	23.2	15.9	30.9	17.9

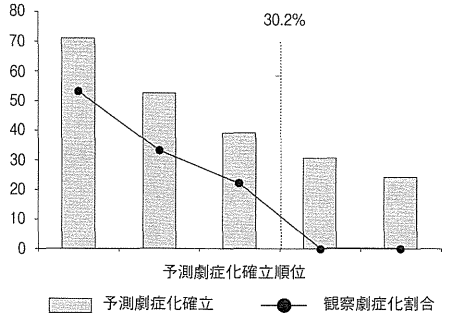

図 2. 劇症化予知精度：Hosmer-Lemeshow test 劇症化予知式による予測劇症化確率 20% 以上（専門施設搬送基準合致例）の症例における，予測劇症化確率（棒グラフ）と観察劇症化割合（折れ線グラフ）との比較を示す．予測劇症化確率 30.2% 未満の症例から，劇症化例は観察されていない．

ティブな検討が行われている．これまでの検討で，予知式の有効性が示されており（図 2），劇症化阻止治療法の探索が進められている．

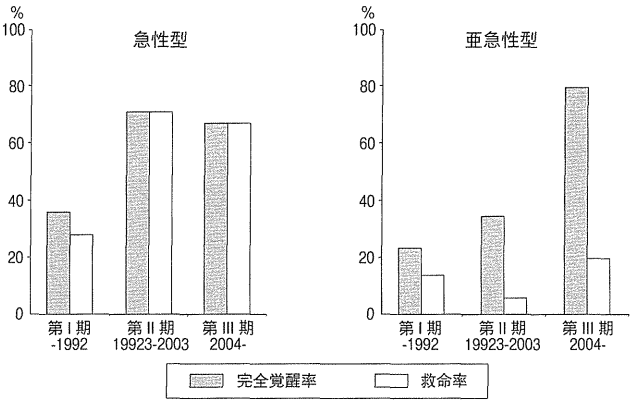

図 3. 人工肝補助の手法の変遷と昏睡覚醒率および救命率との関連
（岩手医大消化器・肝臓内科）
人工肝補助の手法の変遷と昏睡覚醒率，救命率との関連を示す．

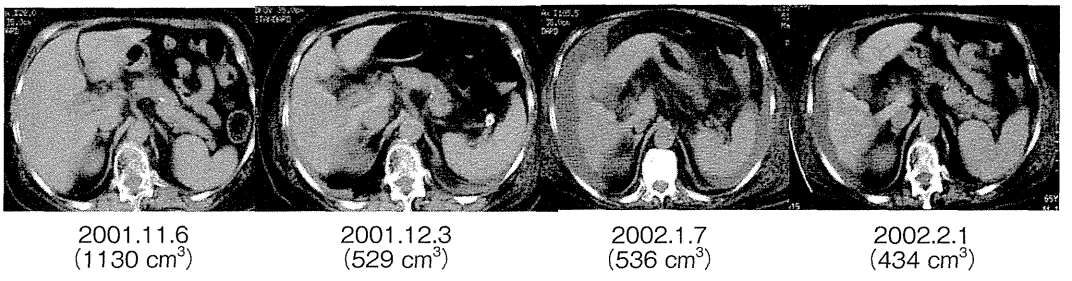

図 4. 劇症肝炎亜急性型例 (65 歳．女性) の腹部 CT の経過
経時的 CT 所見を示す．（ ）内は CT で計測した肝容積を示す．

V. 急性肝不全の標準的治療法

急性肝不全は稀な疾患であり，1 施設での症例数が限られている．しかも急激な経過で生命が危険な状況に陥るため，根本的な治療法についての比較試験が行い難い．このため，肝移植は勿論のこと，人工肝補助療法（ALS）などの主要な治療法に関する無作為化比較試験（RCT）は行われていないのが現状である．しかし，欧米では移植例と内科治療例との救命率の比較から肝移植が救命率を向上する唯一の治療法と考えられている[22]．

これに対し，わが国ではほとんどの全ての症例に ALS（血漿交換＋血液濾過透析）が行われているのが特徴である（表 2）．ALS により肝機能の一部を代償し，意識レベルを維持し，合併症を予防しながら肝の再生を待つのが基本的な治療方針である．そして，内科治療の限界を適切な時期に判断し，肝移植の適応を検討する．わが国では，劇症肝炎の約 25％に肝移植が行われている（図 1）．

我が国の ALS は極めて技術が高く，少なくとも専門施設における昏睡覚醒率および急性型の救命率はめざましく向上した[23]（図 3）．

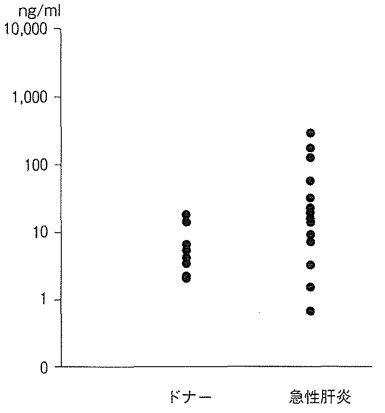

図5. 肝移植ドナーおよび急性肝炎の経過中血清 AFP 最高値
肝移植ドナーは肝切除後7日間の，急性肝炎患者は全入院期間での血清 AFP の最高値を示す．

VI. 肝再生療法の必要性と開発の現状

先に述べたように，ALS を用いた急性肝不全治療の課題は，亜急性型の救命率の向上である．昏睡覚醒率が向上したにもかかわらず，救命率が向上しない原因として，肝再生不全が想定される．図4がその典型例の経過である．この症例は薬物性肝障害（健康食品）による亜急性型急性肝不全例である．断続的な ALS により意識清明に保たれたにもかかわらず，肝の萎縮が進行して救命し得なかった．この例にみられるように，ALS は昏睡覚醒効果は充分だが，肝の再生を促進する効果は不十分と言わざるを得ない．従って，ALS に加えて新たな肝再生促進療法の開発が現在の課題である．

肝は本来再生能の高い臓器である．動物実験では，正常肝では70%の切除でも容易に再生することが古くから知られている．また，日本で広く行われている生体肝移植のドナー手術では最大70%の肝切除が行われるが，急速かつ良好な肝再生を示し，多くの場合1-2週間で退院できる[24]．これに対し，亜急性型急性肝不全では上述のように再生不全を示し[25]肝不全から容易に脱却できない場合が多い．これまで急性肝不全に対し，多くの肝再生療法が提唱されてきたが，臨床的に有効性を示したものはない．現在，ヒト肝細胞増殖因子（HGF）が有力な候補として，実用化を目指した臨床試験が日本で進められている[26]．

正常肝（肝移植ドナー）と急性肝不全にみられる再生像の著しい違いの原因の一つに，再生を担う細胞の違いがあげられる．前者では成熟肝細胞が再生の主体を担うのに対し，後者では肝前駆細胞が主体と考えられている[27]．この臨床的裏付けとして，肝移植ドナーでは血清 alpha-fetoprotein（AFP）が上昇することはほとんどないのに対し，急性肝炎とくに重症肝炎では極めて高い値を示すことがあげられる[28-29]（図5）．このように血清 AFP が高値を示す重症肝炎では，肝前駆細胞のマーカーである CK19 陽性の肝細胞（intermediate hepatocyte）が認められる[30]．

一般的に，成熟肝細胞と肝前駆細胞の至適増殖環境は異なると言われ，両者が同時に増殖することはないと考えられている[31]．従って，急性肝不全における肝局所の微小環境は，肝前駆細胞にとって至適増殖環境にあり，成熟肝細胞の増殖が抑制されていることが再生不全の原因と想定される．以上のことから，成熟肝細胞の至適増殖環境を解明し，この環境を強力な ALS により達成することが，急性肝不全の内科的救命の一つの道と考える．

VII. おわりに

重症肝炎ことに昏睡型急性肝不全の予知・予防，治療の現況，今後の展望を解説した．急性肝炎は無治療で回復する軽症例から，肝移植を要する重症例まで幅広い臨床像を呈する．重症肝炎の克服に向けては，一般施設から専門の治療・研究施設までが一体となって，多方面からの臨床および基礎研究の協力体制が必要と考えられる．

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Short Communication

Combination of acyclic retinoid with branched-chain amino acids inhibits xenograft growth of human hepatoma cells in nude mice

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Aim: Combination chemoprevention is a promising strategy to improve the prognosis of hepatocellular carcinoma (HCC). A malfunction of retinoid X receptor-α (RXR-α) due to phosphorylation by Ras/mitogen-activated protein kinase is closely associated with liver carcinogenesis and acyclic retinoid (ACR) can prevent HCC development by inhibiting RXR-α phosphorylation. The present study examined the possible combined effects of ACR plus branched-chain amino acids (BCAA), which can also prevent the development of HCC in obese patients with liver cirrhosis, in human HCC xenografts in nude mice.

Methods: This study examined the effects of the combination of ACR plus BCAA on the growth of Huh7 human HCC xenografts in nude mice. The effects of the combination on the phosphorylation of RXR-α, extracellular signal-regulated kinase (ERK), Akt and insulin-like growth factor-1 receptor (IGF-1R) proteins, and on the expression levels of retinoic acid receptor-β (RAR-β) and $p21^{CIP1}$ mRNA, were also examined by western blot and real-time reverse transcription polymerase chain reaction analyses, respectively.

Results: The combined treatment with ACR plus BCAA significantly inhibited the growth of Huh7 xenografts. The combination of these agents caused a marked inhibition of the phosphorylation of RXR-α, ERK, Akt and IGF-1R proteins in the xenografts. In addition, the expression levels of RAR-β and $p21^{CIP1}$ mRNA significantly increased by these agents.

Conclusion: The combination of ACR and BCAA restores the function of RXR-α by inhibiting its phosphorylation and increasing the level of RAR-β, a heterodimeric partner for RXR-α, and thus suppresses the growth of HCC xenografts. Therefore, this combination might be an effective regimen for the treatment and, probably, chemoprevention of HCC.

Key words: acyclic retinoid, branched-chain amino acids, hepatocellular carcinoma, phosphorylated retinoid X receptor-α, retinoic acid receptor-β

INTRODUCTION

THE POOR PROGNOSIS for patients with hepatocellular carcinoma (HCC) has created an urgent need to develop more effective strategies for prevention of this malignancy. Retinoids, which have tumor-suppressive and chemopreventive properties in various organs, are considered to be promising agents for improving outcomes in individuals with HCC.[1,2] A clinical trial demonstrated that the administration of acyclic retinoid (ACR), a synthetic retinoid that targets retinoid X receptor-α (RXR-α), significantly reduced the incidence of post-therapeutic recurrence of HCC.[3] ACR inhibits growth in human HCC cells by inducing apoptosis and arrest of the cell cycle in G_0–G_1.[4,5] The inhibition of growth in cancer cells by ACR is also associated with induction of cellular levels of retinoic acid receptor-β (RAR-β), an important retinoid receptor for regulation of apoptosis, and the inhibition of RXR-α phosphorylation.[5–8] The latter effect is more significant because the accumulation of phosphorylated (i.e. inactivated) RXR-α (p-RXR-α) interferes with the function of normal RXR-α in a dominant-negative manner, and therefore plays a critical role in the development of HCC.[2,9,10]

In addition, ACR acts synergistically with various agents (e.g. β-interferon, OSI-461, trastuzumab, valproic

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acid and vitamin K_2) that target other signaling pathways in suppressing growth and inducing apoptosis in human HCC cells.[5,11–14] These findings have clinical significance for the treatment and chemoprevention of HCC because the combined use of two or more agents can diminish drug toxicity while exerting synergistic effects. Branched-chain amino acids (BCAA; leucine, isoleucine and valine), which improve protein malnutrition in patients with liver cirrhosis, are candidate partners in ACR-based combination chemoprevention because a recent clinical trial showed that oral supplementation with these agents reduced the risk of HCC in obese patients with chronic viral liver disease.[15] Treatment with BCAA also prevents the development of liver tumorigenesis in a rodent model, while also inhibiting the growth of HCC cells.[16–18] The purpose of this study is to investigate whether the combination of ACR plus BCAA significantly inhibits the growth of human HCC xenografts and to examine the possible mechanisms of this action.

METHODS

Materials

AN ACYCLIC RETINOID (peretinoin) was supplied by Kowa Pharmaceutical (Tokyo, Japan). BCAA was obtained from Ajinomoto (Tokyo, Japan). The BCAA composition (2:1:1.2 = leucine : isoleucine : valine) was set at the clinical dose used for the treatment of decompensated liver cirrhosis in Japan.[15]

Experimental procedure

Thirty-two male BALB/c nude mice (5 weeks of age) were obtained from Charles River Japan (Tokyo, Japan). Xenograft tumors were made by the s.c. injection of Huh7 human HCC cells (Japanese Cancer Research Resources Bank, Tokyo, Japan) into the flanks of the mice at a concentration of 5×10^6 cells per 200 μL.[19] The mice were randomly divided into four groups (eight mice per group) 1 week after tumor cell injection. The mice in group 2 (ACR alone) were given the basal diet, CRF-1 (Oriental Yeast, Tokyo, Japan), containing 0.03% ACR with free access to feeding for 5 weeks. Group 3 (BCAA alone) was given the basal diet supplemented with 3.0% BCAA (w/w). The mice in group 4 (combination group) received a diet containing 0.03% ACR and 3.0% BCAA. Group 1 was given the basal diet and served as an untreated control. The tumor volume was calculated at the termination of the experiment using the formula: largest diameter × (smaller diameter)^2 × 0.5.

Protein extraction and western blot analysis

Total protein was extracted from the xenografts of Huh7 cells and equivalent amounts of protein (20 mg/lane) were examined by a western blot analysis.[19] The primary antibodies for RXR-α (ΔN-197 and D-20), extracellular signal-regulated kinase (ERK), phosphorylated ERK (p-ERK), Akt, phosphorylated Akt (p-Akt), insulin-like growth factor-1 receptor (IGF-1R), phosphorylated IGF-1R (p-IGF-1R) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) have been previously described.[5,10,19,20] The ΔN-197 antibody is regarded as a specific antibody for the phosphorylated form of RXR-α protein.[8,10] The intensities of the blots were quantified with NIH Image software ver. 1.62.

RNA extraction and quantitative real-time reverse transcription polymerase chain reaction analysis

Total RNA was isolated from the xenografts of Huh7 cells using the RNAqueous-4PCR kit (Ambion Applied Biosystems, Austin, TX, USA). The cDNA was amplified from 0.2 μg of total RNA using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). The primers used for the amplification of RAR-β, $p21^{CIP1}$ and GAPDH-specific genes have been previously described.[6,19] A quantitative real-time RT–PCR analysis was performed in a LightCycler (Roche Diagnostics, Mannheim, Germany) with SYBR Premix Ex Taq (TaKaRa Bio, Shiga, Japan).[16] The gene expression levels were normalized to the GAPDH expression levels using a standard curve.

Statistical analysis

The data are expressed as the mean ± standard deviation. Statistical significance of the difference in mean values was assessed by one-way ANOVA, followed by Scheffé's *t*-test.

RESULTS

Combined treatment with ACR plus BCAA significantly inhibits growth of HCC xenografts

AS SHOWN IN Figure 1, neither treatment with 0.03% ACR alone nor 3.0% BCAA alone inhibited the growth of Huh7 xenografts. These findings suggest that such doses of ACR and BCAA are insufficient to suppress the tumor growth of HCC in the present study, although similar concentrations of these agents have had a significant effect on preventing the development

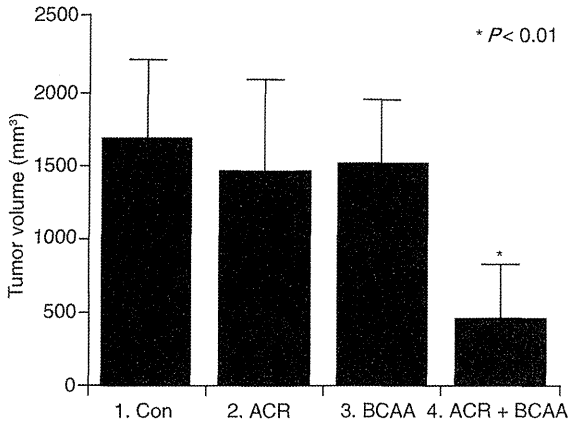

Figure 1 Effects of the combination of acyclic retinoid (ACR) plus branched-chain amino acid (BCAA) on the growth of Huh7 xenografts. BALB/c nude mice were injected s.c. with 5×10^6 Huh7 cells. One week after the injection, the mice were divided into four groups and treated as follows for 5 weeks: group 1, untreated control group (Con); group 2, 0.03% ACR-treated group; group 3, 3.0% BCAA-treated group; group 4, 0.03% ACR and 3.0% BCAA-treated group. The tumor volumes in each group at the termination of experiment are represented. Bars, standard deviation. $*P < 0.01$, significant differences obtained by comparisons to groups 1, 2, and 3.

of HCC in clinical trials.[3,15] On the other hand, the simultaneous treatment of the mice with these concentrations of ACR plus BCAA produced a significant decrease in the growth of HCC xenografts; the tumor volume was inhibited by 73% in the combination treatment group in comparison to the control group ($P < 0.01$).

BCAA inhibits the phosphorylation of Akt and IGF-1R, and enhances the suppression of the RXR-α and ERK phosphorylation by ACR in HCC xenografts

Retinoid X receptor-α phosphorylation by Ras/mitogen-activated protein kinase is closely associated with the development of HCC, and thus might be a critical target for chemoprevention of HCC.[2,9] BCAA inhibits the activation of IGF-1R and Akt and this is associated with the cancer chemopreventive effects of this agent.[16,21] Therefore, the combined effects of ACR plus BCAA on the phosphorylation of RXR-α, ERK, Akt and IGF-1R proteins were investigated in Huh7 xenografts. The expression levels of p-RXR-α and p-ERK proteins, which decreased in the ACR alone-treated group in comparison to the control group (Fig. 2a, column 2), decreased to a greater extent when the mice were treated with the combination of ACR plus BCAA (Fig. 2a, column 4). The expression levels of p-Akt and p-IGF-1R proteins, which were decreased in the BCAA alone-treated group (Fig. 2a, column 3), were also further reduced by combined treatment with ACR plus BCAA (Fig. 2a, column 4).

Combined treatment of ACR plus BCAA induces the RAR-β and $p21^{CIP1}$ mRNA in HCC xenografts

The combined effect of ACR plus BCAA on the induction of the RAR-β and $p21^{CIP1}$ mRNA was next examined because, in addition to the inhibition of RXR-α phosphorylation, ACR is known to inhibit the growth of HCC cells by enhancing the expression of these molecules.[4,5,7] Semiquantitative RT–PCR analyses showed that treatment with both ACR alone and BCAA alone tended to increase the levels of RAR-β mRNA, but the differences were not significant (Fig. 2b, columns 2 and 3). On the other hand, when ACR was combined with BCAA, the expression levels of this mRNA were significantly enhanced in comparison to the control group (Fig. 2b, column 4). In addition, treatment with ACR alone and the combination of ACR plus BCAA significantly increased the expression of $p21^{CIP1}$ mRNA (Fig. 2c, columns 2 and 4), a negative modulator of cell cycle progression,[22] in comparison to the control group.

DISCUSSION

COMBINATION CHEMOPREVENTION IS often advantageous because it provides the potential for additive or, in some instances, synergistic effects between specific agents. The present study clearly indicated that the combination of ACR plus BCAA, both of which exert chemopreventive properties on HCC development,[3,15] causes potent inhibition of growth in human HCC xenografts. The hypotheses that explain this beneficial effect are summarized in Figure 3.

Initially, it should be emphasized that the phosphorylation of RXR-α and ERK proteins was strongly inhibited by the combination of ACR plus BCAA. This study and prior ones[5,8,14,23] show that ACR alone inhibits the phosphorylation of these proteins, thus indicating that BCAA could enhance the effect of ACR in HCC xenografts. These findings seem to be significant because restoration of the function of RXR-α as a master regulator of nuclear receptors by targeting its phosphorylation might be an effective strategy for the prevention and treatment of HCC.[2] BCAA may support the effect of

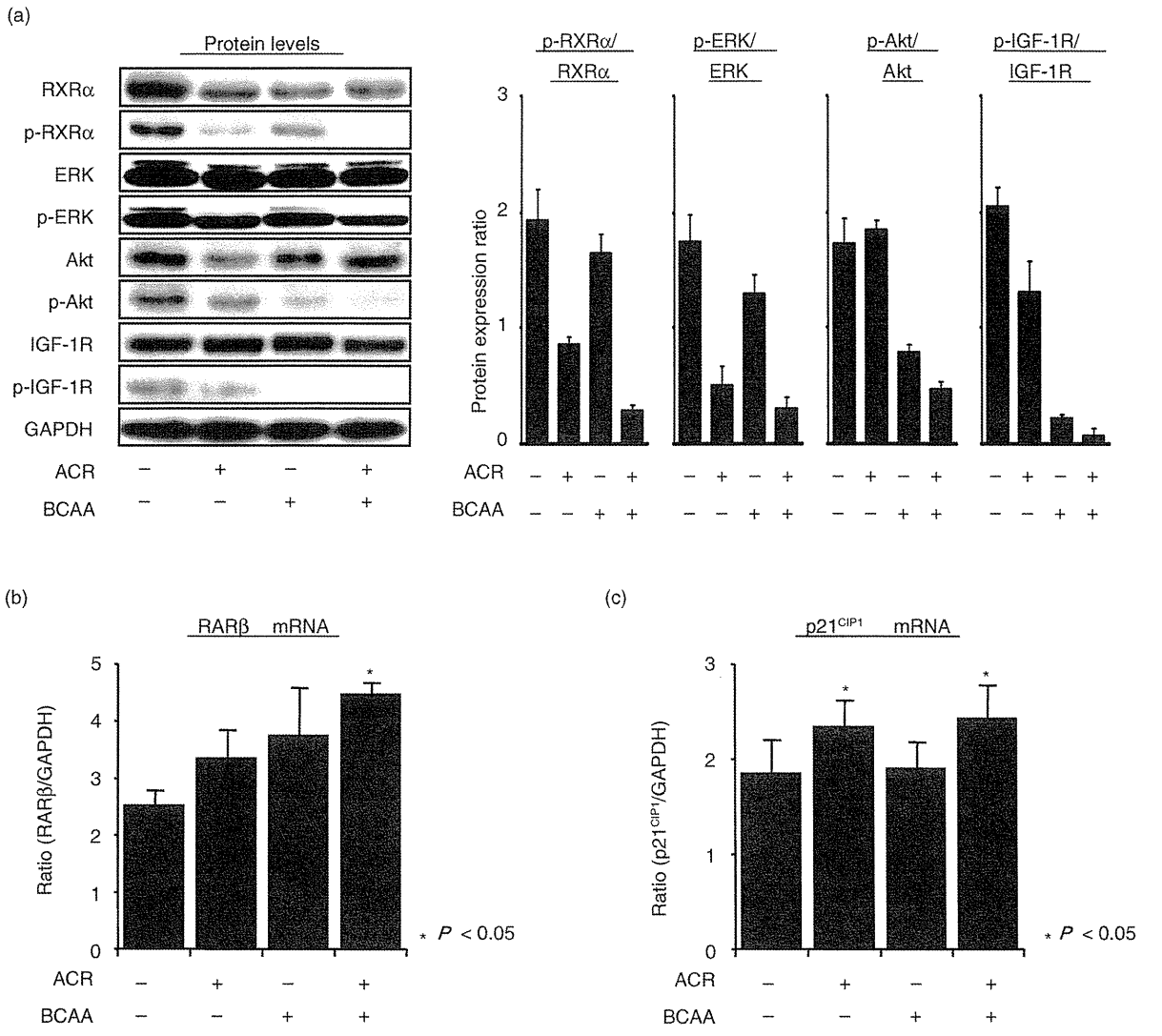

Figure 2 Effects of the combination of acyclic retinoid (ACR) plus branched-chain amino acid (BCAA) on phosphorylation of retinoid X receptor-α (RXR-α), extracellular signal-regulated kinase (ERK), Akt and insulin-like growth factor-1 receptor (IGF-1R) proteins and expression levels of retinoic acid receptor-β (RAR-β) and $p21^{CIP1}$ mRNA in Huh7 xenografts. The xenografts were excised from each animal at the termination of the experiment and tumor extracts were examined by a western blot analysis using the respective antibodies (a) or were examined by a quantitative real-time reverse transcription polymerase chain reaction analysis using RAR-β (b) and $p21^{CIP1}$ (c) specific primers. (a) Western blot analysis for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed using a single membrane and equal protein loading was verified by the detection of this protein. Repeat western blots yielded similar results. Lanes, protein samples from each group (left). The intensities of blots were quantitated by densitometry (right). (b,c) The expression levels of *RAR-β* (b) and *$p21^{CIP1}$* (c) genes were normalized to GAPDH expression. Bars, standard deviations of triplicate assays. *$P < 0.05$, significant differences obtained by comparison to the control group (group 1). p-, phosphorylated.

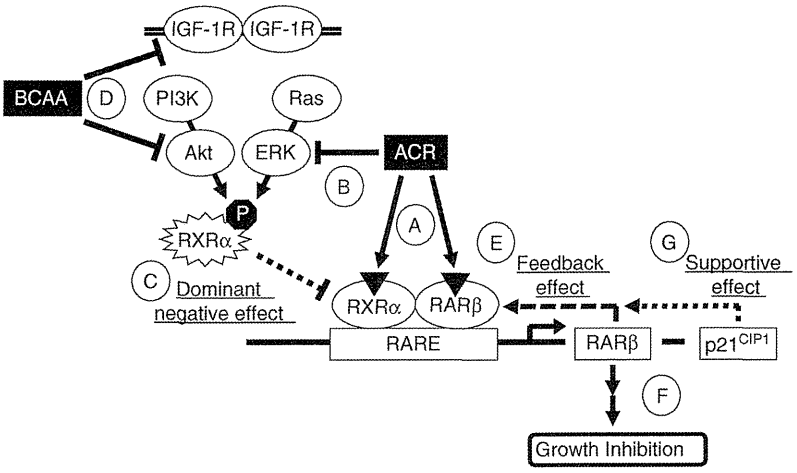

Figure 3 Hypothetical schematic representation of the effect of the combination of acyclic retinoid (ACR) plus branched-chain amino acid (BCAA) on growth inhibition in hepatocellular carcinoma (HCC) xenografts. ACR can bind to both retinoic acid receptor (RAR) and retinoid X receptor (RXR) as a ligand (a), and activate the retinoic acid responsive element (RARE) promoter activity, thus increasing the levels of both RAR-β and $p21^{CIP1}$ because the promoter region of these molecules contains RARE. In parallel, ACR inactivates the Ras/mitogen-activated protein kinase signaling pathways (b). This signaling pathway phosphorylates RXR-α, and thus impairs the function of this receptor in a dominant-negative manner (c). On the other hand, BCAA inhibits the activation of IGF-1R and its downstream Akt, which is also involved in RXR-α phosphorylation (d). Cooperative inhibition of RXR-α phosphorylation by ACR plus BCAA might restore the function of this receptor and subsequently increase RAR-β expression. This induction of RAR-β and its activation by the ligand ACR might produce a positive feedback effect on the expression of RAR-β itself (e), thus enhancing inhibition of growth in HCC cells (f). Induction of $p21^{CIP1}$ might support this positive feedback effect (g). For additional details see the "Discussion" section. ERK, extracellular signal-regulated kinase; IGF-1R, insulin-like growth factor-1 receptor.

ACR, at least in part, by inhibiting the activation of IGF-1R and its downstream Akt, because some types of receptor tyrosine kinases (RTK), including IGF-1R, might phosphorylate RXR-α through the phosphorylation of ERK and Akt. ACR and BCAA reduce the development of HCC and suppress the growth of cancer cells by inhibiting the activation of specific RTK, including IGF-1R and epidermal growth factor receptor (EGFR).[6,16,24] BCAA also suppresses insulin-induced hepatic tumor cell proliferation by inhibiting ERK and Akt phosphorylation.[18] The previous reports showing that there is a cross-talk between EGFR and IGF-1R, and that the simultaneous targeting of these RTK induces a synergistic inhibition of growth in HCC cells, might give this hypothesis credibility.[25,26]

The reduction in the dominant negative effect of RXR-α phosphorylation by combining ACR plus BCAA might activate the transcriptional activity of retinoic acid responsive element (RARE).[5,10] This is associated with the increased expression of RAR-β and $p21^{CIP1}$ mRNA because the promoter region of these genes contains RARE.[27,28] RAR-β, which is also a receptor for ACR, can exert tumor-suppressive effects in cancer cells.[29] Therefore, the induction of RAR-β by the treatment with ACR plus BCAA might have played a critical role in inhibiting the growth of HCC xenografts in the present study. In addition, this induction might be, at least in part, associated with $p21^{CIP1}$ upregulation by ACR plus BCAA because introduction of the *$p21^{CIP1}$* gene into cells induces RAR-β expression and sensitizes cancer cells to retinoid treatment.[30] This hypothesis may be supported by recent reports that a substantial induction of RAR-β and $p21^{CIP1}$ produces positive feedback effects on the expression of RAR-β.[5,12]

Acyclic retinoid has an agonistic activity for both RAR and RXR.[2] Therefore, the reduction of the dominant-negative effect of RXR-α phosphorylation and the induction of the RAR-β expression by the combination of ACR plus BCAA might exert a significant inhibition of growth in the HCC xenografts. Because this study shows the possibility that the combination treatment consisting of ACR plus BCAA is an effective regimen for the treatment of HCC, we presume that this combination might also be useful for the prevention of HCC. In order to confirm

this prediction, future studies are required to determine whether this combination treatment prevents the development of HCC using chemically-induced liver carcinogenesis in a rodent model with, for example, diethylnitrosamine.

In conclusion, this study, as well as prior ones,[5,11–14] indicates that the combination chemoprevention using ACR as a key agent might be an effective strategy for the prevention and treatment of HCC. Among such regimens, particularly combining ACR with BCAA might hold promise as a clinical modality for the chemoprevention of HCC because clinical trials have shown that both of these agents can significantly prevent the development of HCC without causing any adverse effects.[3,15]

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Preventive effects of branched-chain amino acid supplementation on the spontaneous development of hepatic preneoplastic lesions in C57BL/KsJ-*db/db* obese mice

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Obesity and its associated disorders, such as non-alcoholic steatohepatitis, increase the risk of hepatocellular carcinoma. Branched-chain amino acids (BCAA), which improve protein malnutrition in patients with liver cirrhosis, reduce the risk of hepatocellular carcinoma in these patients with obesity. In the present study, the effects of BCAA supplementation on the spontaneous development of hepatic premalignant lesions, foci of cellular alteration, in *db/db* obese mice were examined. Male *db/db* mice were given a basal diet containing 3.0% of either BCAA or casein, a nitrogen-content-matched control of BCAA, for 36 weeks. On killing the mice, supplementation with BCAA significantly inhibited the development of foci of cellular alteration when compared with casein supplementation by inhibiting cell proliferation, but inducing apoptosis. BCAA supplementation increased the expression levels of peroxisome proliferator-activated receptor-γ, $p21^{CIP1}$ and $p27^{KIP1}$ messenger RNA and decreased the levels of c-*fos* and cyclin D1 mRNA in the liver. BCAA supplementation also reduced both the amount of hepatic triglyceride accumulation and the expression of interleukin (IL)-6, IL-1β, IL-18 and tumor necrosis factor-α mRNA in the liver. Increased macrophage infiltration was inhibited and the expression of IL-6, TNF-α, and monocyte chemoattractant protein-1 mRNA in the white adipose tissue were each decreased by BCAA supplementation. BCAA supplementation also reduced adipocyte size while increasing the expression of peroxisome proliferator-activated receptor-α, peroxisome proliferator-activated receptor-γ and adiponectin mRNA in the white adipose tissue compared with casein supplementation. These findings indicate that BCAA supplementation inhibits the early phase of obesity-related liver tumorigenesis by attenuating chronic inflammation in both the liver and white adipose tissue. BCAA supplementation may be useful in the chemoprevention of liver tumorigenesis in obese individuals.

Introduction

Obesity is a serious health problem worldwide since it often causes a number of medical disorders, including metabolic syndrome and type 2 diabetes mellitus. Recent evidence also indicates that obesity and its related metabolic abnormalities are associated with an increased risk of developing hepatocellular carcinoma (HCC (1–5)). Non-alcoholic fatty liver disease (NAFLD) is a hepatic manifestation of metabolic syndrome and a subset of patients with this disease can progress to non-alcoholic steatohepatitis (NASH), which involves the risk of developing chronic hepatitis, cirrhosis and HCC (6–8). Obesity and diabetes mellitus have been shown to increase the risk of developing HCC also in patients with viral hepatitis (3,5). A state of chronic inflammation caused by insulin resistance and hepatic steatosis is considered to play a critical role in the development of HCC in several obesity-related pathophysiological conditions (2,6–10). Therefore, obese patients, especially those with complications of NASH or chronic viral hepatitis, are at high risk for developing HCC, and targeting chronic inflammation might be an effective strategy for preventing obesity-related liver carcinogenesis (11).

Branched-chain amino acids (BCAA), which are a group of three essential amino acids comprising valine, leucine and isoleucine, are used clinically to improve protein malnutrition in patients with liver cirrhosis (12,13). Oral supplementation with BCAA prevents progressive hepatic failure and improves event-free survival in patients with chronic liver diseases (14,15). Moreover, a multicenter, randomized controlled trial has reported that long-term oral BCAA supplementation reduced the risk of developing HCC in patients with chronic viral hepatitis; however, the effect was evident only in the patients who are obese (3). The results seen in that clinical trial are considered to be associated with the improvement of insulin resistance achieved by BCAA supplementation (13,16). In fact, BCAA supplementation inhibited the development of carcinogen-induced liver and colorectal carcinogenesis in obese mice by improving insulin resistance (17,18). Treatment with BCAA also suppressed insulin-induced proliferation of HCC cells by antagonizing the anti-apoptotic function of insulin (19).

In addition to improving protein malnutrition and glucose metabolism, BCAA supplementation has been reported to reduce lipid deposition in the liver in recent rodent studies (17,20). Supplementation with BCAA also retarded excess weight gain and reduced epididymal white adipose tissue (WAT) weight in mice that fed a high-fat diet (20). Because chronic low-grade systemic inflammation produced by excess lipid storage in WAT and liver is involved in both the development of NASH and the obesity-related liver tumorigenesis (2,6–10), BCAA supplementation may prevent the development of liver neoplasms in obese mice by reducing excess fat accumulation in WAT and by improving liver steatosis, thereby attenuating inflammation in these organs.

The spontaneous development of hepatic preneoplastic lesions, foci of cellular alteration (FCA), have been previously reported to be enhanced in obese and diabetic C57BL/KsJ-*db/db* (*db/db*) mice, when compared with C57B6 or C57BL/KsJ-+/+ mice, genetic controls for *db/db* mice (17). In the present study, we examined the effects of BCAA supplementation on the spontaneous development of FCA in *db/db* mice while focusing on the attenuation of inflammation in both the liver and the WAT. In addition, we investigated whether BCAA supplementation alters adipocyte size and the expression of peroxisome proliferator-activated receptor (PPAR)-α, PPAR-γ and adiponectin, which are key regulators of inflammatory signaling in obese adipose tissue (21–25), in the WAT of *db/db* mice.

Materials and methods

Mice and diets

Male *db/db* mice (4 weeks old) were obtained from Japan SLC (Shizuoka, Japan) and humanely maintained at Gifu University Life Science Research Center in accordance with Institutional Animal Care Guidelines. BCAA and casein were obtained from Ajinomoto (Tokyo, Japan). The BCAA composition (2:1:1.2 = leucine:isoleucine:valine) was set at the clinical dosage used for the treatment of decompensated liver cirrhosis in Japan (3,14).

Abbreviations: BCAA, branched-chain amino acids; FCA, foci of cellular alteration; HCC, hepatocellular carcinoma; H&E, hematoxylin and eosin; IL: interleukin; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PCNA, proliferating cell nuclear antigen; PPAR, peroxisome proliferator-activated receptor; RT–PCR, reverse transcription–PCR; SEM, standard error mean; TNF-α, tumor necrosis factor-α; WAT, white adipose tissue.