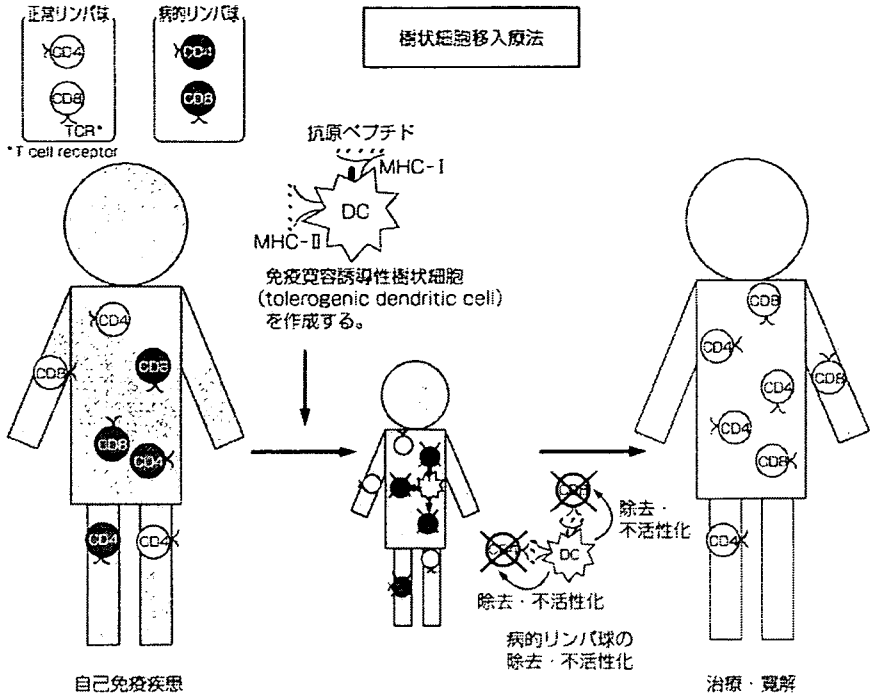


図❷ 樹状細胞の移入療法による自己免疫疾患の治療
自己免疫疾患における細胞移入療法として，樹状細胞移植療法を示す．樹状細胞移植療法は，免疫寛容能をもった樹状細胞により，病的リンパ球のみを除去・不活性化して治療効果を発現する．

表❶ これまでに報告されている樹状細胞（DC）移入療法の概要

樹状細胞（DC）移入療法	免疫抑制の機序	参考文献
未熟 DC の移入療法	IL-10 産生 CD8 陽性 T 細胞の誘導（ヒト *in vivo* での報告）	4)
培養法の調整による抑制性 DC の作成		
低濃度 GM-CSF による培養	DC の共刺激分子の発現抑制，成熟抑制	5)
TGF-β を用いた培養	DC の成熟抑制	6)
IL-10 を用いた培養	DC の共刺激分子および MHC クラス II 分子の発現抑制	7)
TNF-α による成熟刺激	IL-10 産生 CD4 陽性 T 細胞の誘導	8)
抗 CD40 抗体と DC の同時移入療法	制御性（regulatory）T 細胞の誘導？	9)
遺伝子導入による抑制性 DC の作成		10)
IL-10	DC の共刺激分子および MHC クラス II 分子の発現抑制	11)
FasL	活性化 T 細胞の除去	12)
IDO（Indoleamine 2,3-dioxygenase）	活性化 T 細胞の除去	13)
TRAIL	活性化 T 細胞の除去	14)
遺伝子改変 ES 細胞由来の樹状細胞の誘導	活性化 T 細胞の抑制・除去（本文参照）	15)～17)

1）未熟樹状細胞の移入による抗原特異的免疫抑制

未熟樹状細胞は，T 細胞にアナジーあるいは細胞死を誘導する活性を有していると考えられている．そこでヒトの末梢血から *in vitro* で樹状細胞を分化誘導し，成熟刺激を加えない“未熟”な状態でモデル抗原としてインフルエンザウイルスに由来するペプチドをパルスし，人体に戻すという試みがおこなわれた．この結果，投与する前には認められていた，このペプチドに対する T 細胞応答のみを特異的に抑制できることが示された[4]．また，この場合の免疫抑制の機序として，未熟樹状細胞により IL-10 を産生する CD 8 陽性 T 細胞が誘導されていることが示された．

2）特殊な培養法による抑制性樹状細胞の誘導

骨髄などから樹状細胞を誘導する際には顆粒球マクロファージコロニー刺激因子（granulocyte-macrophage colony stimulating factor：GM-CSF）が必要であるが，通常用いるよりも低濃度のGM-CSFで誘導したり，あるいは通常濃度のGM-CSFで誘導した骨髄由来の樹状細胞を抗炎症性サイトカインであるトランスフォーミング増殖因子-β（transforming growth factor-β：TGF-β）やインターロイキン（IL）-10を培養終末に添加することにより，CD 80やCD 40などの共刺激分子の発現が低い“未熟な”樹状細胞ができることが明らかになった．これらの樹状細胞を用いた *in vitro* での実験においてallo MLR（mixed lymphoid reaction）を抑制し，さらに *in vivo* においてもこの樹状細胞をマウスに投与することにより，免疫寛容能を誘導した系統に特異的に移植片の長期間の生着が認められた[5)~7)]．

また *in vitro* でマウスの樹状細胞の成熟を誘導する場合には，腫瘍壊死因子-α（tumor necrosis factor-α：TNF-α），抗CD 40抗体，リポ多糖（lipopolysaccharide：LPS）などで刺激を与えることが多いが，TNF-αのみで刺激した樹状細胞はMHCクラスIIや共刺激分子は高発現するにもかかわらず，IL-10やIL-12の産生が少ない．このように不完全な成熟を示した樹状細胞（semi-mature DC）は，*in vivo* において抗原特異的に免疫応答を抑制し，実験的自己免疫（アレルギー）性脳脊髄炎（experimental autoimmune encephalomyelitis：EAE）の発症を抑制することが報告されている．この *in vivo* での反応は，不完全成熟樹状細胞によりIL-10を産生するCD 4陽性T細胞が誘導されることと関連があるようであるが，その詳細な機構は不明である[8)]．

以上より，樹状細胞を種々の特殊な培養方法で調整することにより，免疫寛容能を獲得した樹状細胞を誘導することができ，これを用いて，抗原特異的な免疫抑制状態を誘導できることが動物実験において示されている．

3）樹状細胞と抗CD 40抗体の同時移入による免疫抑制

樹状細胞はCD 40を発現し，活性化T細胞に発現するCD 40リガンド（CD 154）との結合により，樹状細胞の成熟が促進されることが知られている[9)]．マウスの心臓移植実験において，CD 40に対する阻害抗体（機能を阻害する抗体）とアロ樹状細胞をともに投与することにより，アロ特異的に心臓移植の長期生着が認められた．一方，共刺激分子であるCD 80やCD 86に対する阻止抗体を同様に用いても，移植心臓の長期生着は認められなかった．また，長期移植できたマウスの脾臓細胞を養子移植（adoptively transfer）したところ，レシピエントマウスにおいても移植心臓の長期生着が観察された．以上のことから，*in vivo* において，阻害抗体によりCD 40をブロックされた樹状細胞が，アロの移植片に対する免疫寛容を積極的に誘導するT細胞を誘導している可能性が示唆されている．

3．遺伝子改変による樹状細胞の機能修飾

免疫応答を抑制することが報告されているさまざまな分子〔IL-10, FasL, indoleamin 2,3-dioxygenase（IDO），TNF-related apoptosis-inducing ligand（TRAIL）など〕を，遺伝子銃（gene gun）やアデノウイルスあるいはレトロウイルスなどを用いて遺伝子導入して，樹状細胞に発現させることにより，免疫抑制能を強化した免疫寛容誘導性（tolerogenic）樹状細胞を作製する研究が報告されている[10)]．

IL-10遺伝子を導入した樹状細胞は，MHCクラスIIや共刺激分子の発現が低く，アロ反応性T細胞の活性化を抑制する．これは，IL-10が樹状細胞の成熟を抑制することにより免疫抑制作用を発現するものと考えられる．また，IL-10がTh 1/Th 2のバランスをTh 2へシフトさせることも関連していると考えられる[11)]．

正常細胞や癌細胞，活性化T細胞などをアポトーシスに導くことが知られている *FasL*（*CD95L*）遺伝子を骨髄由来単球（BM-Mono）や骨髄由来樹状細胞に導入し，FasLを高発現した樹状細胞が作製されている．これらの樹状細胞は，*in vitro* と *in vivo* においてアロ抗原に特異的な免疫応答を抑制した．さらに，この樹状細胞に抗原（卵白アルブミン：OVA）を貪食させたものを用いて，*in vivo* においてOVA抗原特異的な免疫抑制を誘導す

ることが可能であった．これは，樹状細胞に反応するアロ抗原特異的あるいは非自己抗原（OVA）特異的T細胞が，樹状細胞により活性化され，これに伴いFasの発現が高まることでFasLへの感受性が増し，アポトーシスが誘導されるためと考えられている[12]．

IDOは，トリプトファンを代謝する酵素であるが，T細胞にアポトーシスを誘導する活性があり，妊娠時の胎児に対する免疫寛容に関与していることが指摘されている．最近，ヒト末梢血の単球より *in vitro* で樹状細胞を作製し，これにアデノウイルスベクターを用いてIDOを発現させ，T細胞応答を抑制する試みが報告されている．この報告によると，IDO発現樹状細胞により，抗CD3抗体刺激によるT細胞増殖反応とアロ抗原に対するT細胞増殖反応が抑制され，この抑制はT細胞のアポトーシスを伴っていた．さらに，B細胞およびナチュラルキラー（natural killer：NK）細胞もIDOにより細胞死が誘導された．一方，IDOは樹状細胞自身には影響を与えなかった[13]．

TRAILは，TNFファミリーであるが，T細胞にアポトーシスを誘導する活性が報告されている．最近，マウスの骨髄由来樹状細胞にアデノウイルスを用いて遺伝子導入し，TRAILを高発現する樹状細胞が作製されている．この樹状細胞にマウスの関節炎を誘導するII型コラーゲンを付加して貪食・抗原提示させ，マウスに投与したところ，そのII型コラーゲンによる関節炎（collagen II induced arthritis：CIA）の発症が抑制された．これは活性化されたII型コラーゲン特異的な病原性T細胞が，II型コラーゲン由来のペプチドを抗原提示しTRAILを高発現する樹状細胞によってアポトーシスを誘導されるためと考えられている[14]．

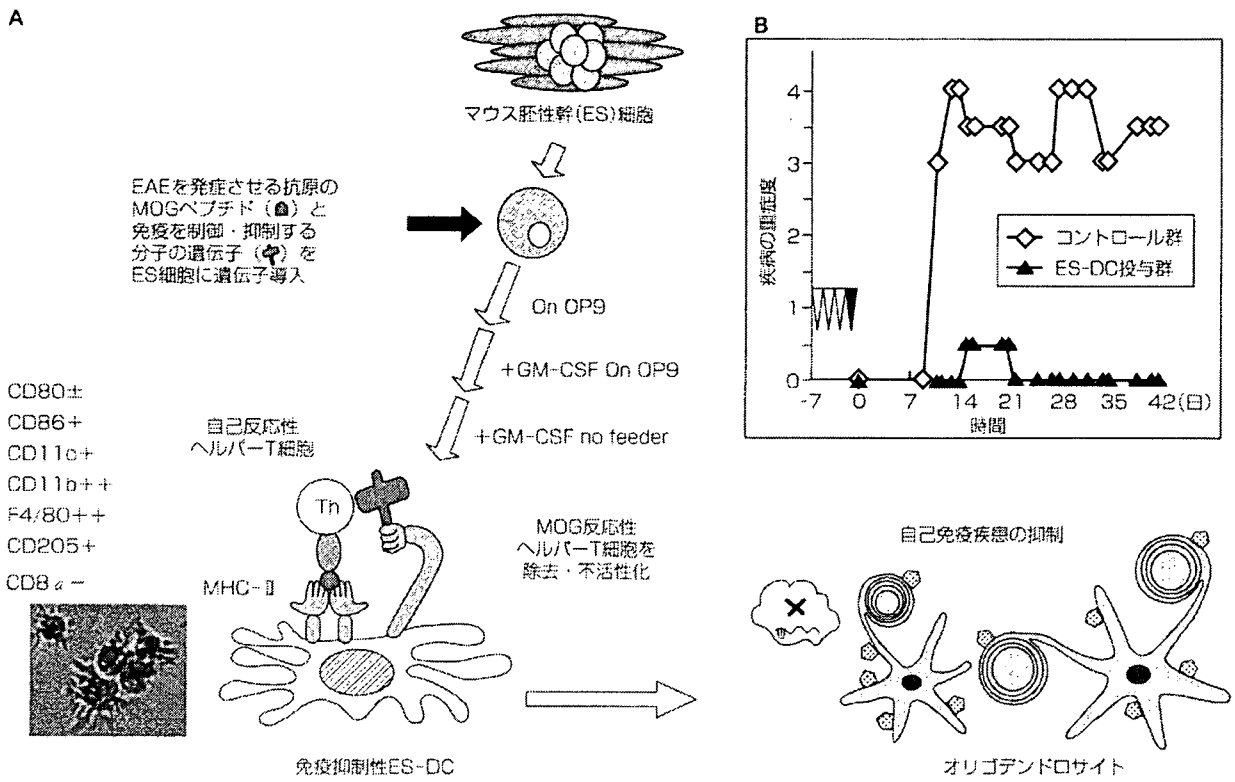
このほかにも，さまざまな免疫制御分子をコードする遺伝子が樹状細胞に導入され，アポトーシス，アナージー，Th1/Th2バランスの制御を介して，アロ抗原あるいは非自己抗原に特異的に免疫寛容を誘導したモデルが示されている．

また，サイトメガロウイルスが樹状細胞に感染すると，感染樹状細胞にFasLやTRAILなどを発現させ，宿主の免疫系による排除から逃れているという報告や，種々の腫瘍細胞はTGF-β，PD-L1などを高発現して免疫系から回避しているとの報告がある．これらの事実は，このようなT細胞応答を抑制する分子が実際に抗原特異的な免疫抑制を引き起こすことが可能であることを強く示唆し，また免疫抑制の方策を考案するうえで新たなヒントを与えてくれるものである．

4．ES細胞由来の樹状細胞を用いた免疫制御

樹状細胞に遺伝子改変をおこなう方法として，これまでは，レトロウイルスあるいはアデノウイルスなどのウイルスベクターを用いる方法が主流であった．しかし，ウイルスベクターを用いる方法には，遺伝子導入の効率と安定性，さらにはベクターシステムのもつ潜在的な危険性などの問題が伴う．そこで，われわれはウイルスベクターを使用しなくても，電気穿孔法により容易に遺伝子導入が可能なES細胞に着目し，これに遺伝子導入した後に樹状細胞に分化誘導する方法を開発した．これまでに，マウスES細胞よりOP9〔macrophage-colony stimulating factor（M-CSF）を産生しないマウス骨髄のストローマ細胞〕とGM-CSFを用いて，樹状細胞（ES-DC）を分化誘導する方法を確立している．このES-DCは，細胞表面にCD11c，CD40，CD80，CD86，CD205，F4/80を発現し，貪食能や抗原提示能などをもち，機能的樹状細胞であることが確かめられている．さらに，ES細胞に任意の抗原遺伝子を遺伝子導入し，これを樹状細胞に分化誘導したところ，*in vitro* において，このES-DCはMHC上に抗原ペプチドを提示していることが確認された．そして，この抗原遺伝子導入ES-DCをマウスへ投与することにより，抗原特異的にT細胞を活性化できた[15][16]．

この手法を応用して，ES細胞にEAEを誘導するミエリン蛋白質の一種であるmyelin oligodendrocyte glycoprotein（MOG）由来のペプチドをコードする遺伝子と，免疫応答を制御・調節する機能を有するさまざまな分子の遺伝子を導入し，それを樹状細胞へ分化誘導し，導入抗原ペプチド＋MHCクラスIIとリンパ球制御因子を細胞表面に高発現したES-DCを作成することができた．この遺伝子改変ES-DCを前投与することにより，



図❸ 遺伝子改変 ES-DC による EAE（実験的自己免疫性脳脊髄炎）の発症予防（Hirata S *et al*, in press[17]より改変引用）
A：EAE を発症させる抗原として知られている MOG ペプチドをコードする遺伝子と，免疫を制御・抑制することが報告された分子の遺伝子を ES 細胞に遺伝子導入し，これを樹状細胞（ES-DC）へ分化誘導した．
B：この遺伝子改変 ES-DC（▲）を 1×10^6ずつ 3 回前投与することにより，ES-DC を投与していない群（コントロール群）（◇）に比較して，MOG ペプチドで誘導される EAE の発症が抑制された（▽は ES-DC の前投与，▼は EAE の誘導を示す）．

MOG ペプチドで誘導される EAE の発症を抑制することができ，さらにこの ES-DC を投与しても無関係な抗原（KLH）に対する免疫応答への影響は観察されず，抗原特異的免疫抑制が誘導されていることを示した（図❸）[17]．

この方法は，従来のアデノウイルスなどを用いた骨髄樹状細胞への遺伝子導入と比較して，電気穿孔法で遺伝子導入するためウイルスを用いることによる危険性を回避でき，また ES 細胞の増殖能により 1 度クローンを確立するとそれぞれの導入遺伝子を安定して発現した樹状細胞を永続的，大量に供給することができる．したがって遺伝子改変 ES-DC は，一般に寛解と再発をくり返す慢性疾患である自己免疫疾患の治療・寛解の維持に有効である可能性があると考えられる．なお，これまでの研究では，ES-DC の無限増殖や，マウスに未分化な ES 細胞を投与した後に発生する奇形腫（teratoma）の形成は観察されていない．現時点ではヒト ES 細胞の利用についての倫理的問題が論議されているが，将来的には倫理的・技術的な諸問題を解決できれば，ヒト ES-DC の免疫療法への応用が期待される．

5．樹状細胞による制御性 T 細胞の誘導

最近，T 細胞の増殖やサイトカイン産生を抑制する制御性 T 細胞の表面分子マーカーの解析がすすみ，この細胞の性質が詳しく研究されてきている．この制御性 T 細胞を大量にマウスに養子移植すると，レシピエントマウスは MOG ペプチドで誘導される EAE の発症を抑制することが報告されている[18]．また，樹状細胞が *in vitro* や *in vivo* において制御性 T 細胞に増殖反応を誘導できる

ことが明らかとなった[3]．このため，抗原特異的な制御性T細胞を *in vitro* で樹状細胞を用いて増殖させ，抗原特異的な免疫抑制をおこなう研究が試みられている．これまでに，ヒトI型糖尿病の動物モデルであるNODマウスの実験系で，膵島由来の抗原を認識するT細胞レセプターをトランスジェニックしたマウスから制御性T細胞を分離し，*in vitro* で抗原蛋白と樹状細胞で増殖させて，これをNODマウスへ移植したところ，発症を予防できたとの報告がある[19,20]．また，実用化のためには多くの課題が残されているが，将来の自己免疫疾患の治療における新たなストラテジーとして，更なる研究が期待される．

おわりに

自己免疫疾患に対する樹状細胞を用いた治療法は，まだ研究段階にあり，樹状細胞を用いた抗原特異的な免疫制御療法については，現時点では，おもにモデル抗原を用いた動物実験により有効性やその安全性の確認をおこなっている段階にある．しかしながら，原因に対する治療法が確立されていない自己免疫疾患に対する治療法開発への新たな戦略の1つとして，今後の更なる研究開発が期待される．われわれは，抗原特異的な免疫制御療法の開発は，免疫学に課せられた重要な課題として解決すべき問題であると考えている．

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瀉血療法

佐々木 裕*
さ さ き ゆたか

● 瀉血療法は，C型慢性肝炎の進行を抑制する肝庇護療法の一つである．
● 肝臓の鉄過剰状態による酸化ストレス状態が肝細胞を障害し線維化や発癌をもたらす．
● 瀉血療法は簡便で安全でかつ有効な治療法である．
● 瀉血療法に鉄制限食を併用する除鉄療法が重要である．

Key words: C型慢性肝炎，瀉血療法，鉄制限食，酸化ストレス，発癌

現在，本邦におけるC型慢性肝炎の治療においては，インターフェロン療法がウイルス排除を目指した唯一の根本的治療法である．ペグインターフェロンとリバビリンの併用療法により，1型高ウイルス量であってもウイルス排除率は約50%と大きく向上した．しかしインターフェロン無効例，年齢，副作用，うつ病などの基礎疾患を有するインターフェロン治療困難例，また通院頻度や経済的理由により治療継続が困難な症例が少なからず存在する．一方，抗ウイルス効果はないが，血清ALT（alanine aminotransferase）値を改善させて肝炎の進行を抑制し肝硬変，肝細胞癌への進展を阻止するための肝庇護療法がある．肝庇護療法には，グリチルリチン製剤である強力ミノファーゲンCの注射，ウルソデオキシコール酸（UDCA）の内服，小柴胡湯などの漢方薬の内服がある．

2006年4月，インターフェロンや肝庇護療法に抵抗性のあるC型慢性肝炎に対する瀉血療法が保険適応となり，わが国におけるすべての医療施設において保険診療として施行することが可能となり，瀉血療法が肝庇護療法の中のもう一つの有力な選択肢となった．

□ 酸化ストレスと肝障害

薬剤，アルコール，C型肝炎ウイルス感染による炎症性サイトカインなどの外因性刺激や，肝細胞内への過剰な鉄や脂肪の沈着による内因性刺激により肝細胞での活性酸素種（reactive oxygen species：ROS）の産生が亢進する．特に肝臓の代謝活動に必要なエネルギーには，おもに体内に取り込まれた酸素が肝細胞のミトコンドリア（Mt）呼吸鎖で酸化的リン酸化に利用される際に産生されるアデノシン三リン酸（ATP）が使用される．この過程の中でROSの発生をともなう．ROSの産生が亢進し過剰な状態が酸化ストレスとなる．ROSにはスーパーオキサイド（O_2^-），過酸化水素（H_2O_2），ヒドロキシラジカル（・OH），一酸化窒素（NO）などが含まれるが，そのなかでもフリーラジカルである・OHはもっとも組織障害性が強い．

一方，食事に含まれる鉄は，十二指腸および上部空腸の腸管上皮細胞からDMT1（divalent metal transporter 1）により吸収され，ferroportin 1により血中に放出される．その一部の鉄が，肝臓でトランスフェリン受容体1（TfR1）を介して取り込まれる．慢性C型肝炎患者では，肝細胞内への鉄の取り込みに関与しているTfR1の発現が亢進している．また肝臓由来の鉄吸収調節ホルモンであるヘプシジンは，十二指腸および上部空腸での鉄吸収抑制作用を有しているが，慢性C型肝炎患者では，血清中のプロヘプシジンが低下傾向であり，腸管での鉄吸収の増加も生じ[1]，結果的に肝臓は鉄過剰状態となっている．肝臓は生体内での最大の鉄貯蔵庫であるが，通常，細胞内では有害な遊離鉄イオンとしてではなく，

*熊本大学医学部 消化器内科

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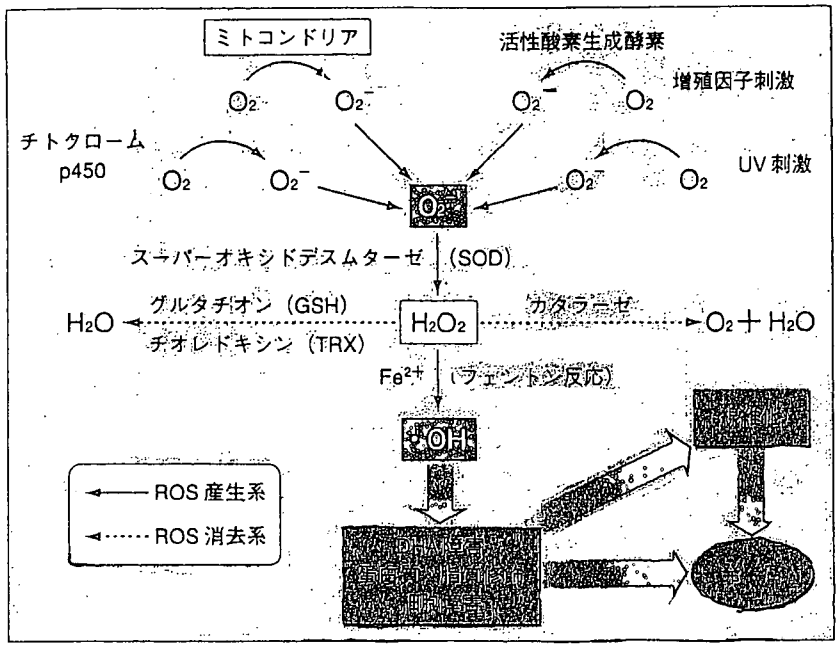


図1
活性酸素種（ROS）の産生と酸化ストレス

大部分がフェリチンやヘモジデリンによって貯蔵されている．しかし肝臓での鉄過剰状態により遊離鉄イオンである Fe^{2+} が発生し，その存在下で酸素から O_2^- が発生し H_2O_2 を介してフリーラジカルである・OH が産生される（フェントン反応）．

慢性C型肝炎では，肝臓での鉄過剰状態によりフリーラジカルである・OH 産生が亢進し，過剰に産生された・OH により肝細胞が障害され，ひいては肝の線維化や発癌がもたらされる（図1）．このような酸化ストレス状態はC型慢性肝炎の病態の一端と考えられている[2]．

□ 瀉血療法

瀉血はその名の通り体内から血液を除去し，人工的に一時的な鉄欠乏性貧血にすることで骨髄での赤血球の造血作用を促進させる．そしてヘモグロビン合成のために肝臓の過剰な鉄が骨髄へ動員され，結果的に酸化ストレスによる肝障害を改善させる理にかなった治療法である．

生体内には通常 4〜6 g の鉄が存在し，その約65%が赤血球内ヘモグロビン鉄である．血液 1 m*l* あたり約 1 mg の鉄が含まれており 1 回 200〜400 m*l* の瀉血で 0.2〜0.4 g の鉄を除去できることになる．

1994 年，Hayashi らによりC型慢性肝炎患者に対して国内で初めて瀉血療法が行われ，血清 ALT 値が有意に改善されたと報告された[3]．その後 2006 年 4 月，瀉血療法が保険適応となり現在までに国内外でも多数の施設からの瀉血療法の治療効果について報告されている[4,5]が，重篤な副作用の報告はなく，瀉血療法は簡便で安全でかつ有効な治療法であることが証明されている．

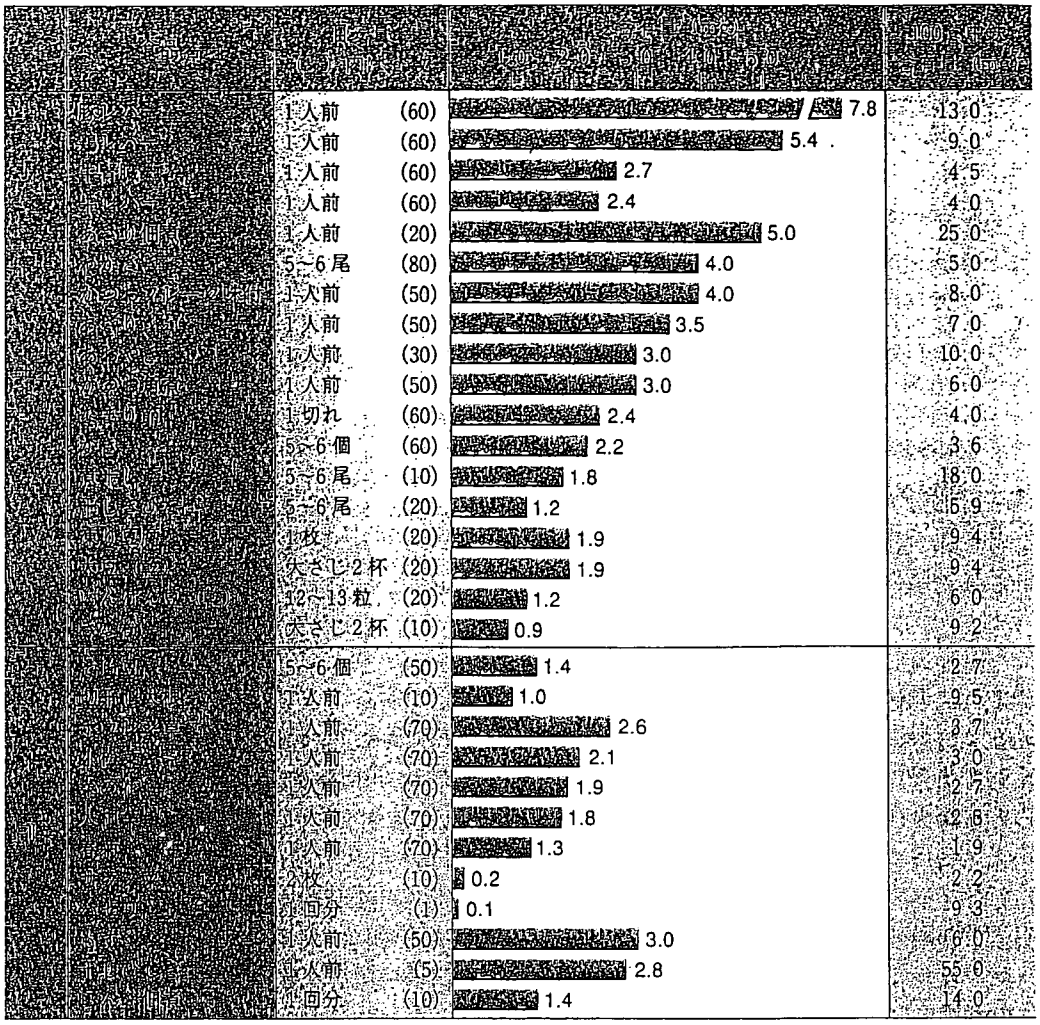
1．瀉血療法の適応

C型慢性肝炎症例のなかで，インターフェロン治療が無効や困難であり，かつ他の肝庇護療法では治療効果が不十分あるいは継続困難な症例が適応となる．基礎疾患に貧血（ヘモグロビン 11.0 g/d*l* 以下）がなく，心疾患，腎不全，呼吸不全の症例は除外する．

2．瀉血療法の方法

一般的な瀉血療法の方法は，2〜4 週間ごとに 1 回あたり 200〜400 m*l* の瀉血を行い，血清フェリチン 10 ng/m*l* を目標に，あるいはヘモグロビン値 11 g/d*l* 以下になるまで繰り返す．治療中，男性でヘモグロビン値 10 g/d*l* 以下，女性でヘモグロビン値 9 g/d*l* 以下の場合はいったん瀉血を中止し造血能の回復を待つ．目標値到達後は，フェ

表1　鉄を多く含む食品



リチン値を10～20 ng/m*l*に保つようにしながら，適宜瀉血の間隔を延長していく．また瀉血した血液はHCV感染血液であることから，取り扱いには十分注意が必要である．

副作用としては，瀉血後に一過性の易疲労感や動悸などの貧血症状を認める場合や，循環血漿量減少による迷走神経反射のため徐脈や血圧低下を認め，補液を必要とする場合がある．そのため仰臥位にて瀉血を行うようにする．また頻回な瀉血によって低アルブミン血症をきたし浮腫を認めることもあるが，瀉血の中止により改善する．その他特別な処置を必要とするような重篤な副作用の報告はない[6]．

3．鉄制限食

瀉血療法の効果を維持していくために鉄制限食の指導も重要である．瀉血療法により鉄欠乏性貧血の状態であるため，十二指腸および上部空腸からの鉄吸収が亢進してしまうためである．瀉血療法に鉄制限食を併用するいわゆる除鉄療法はきわめて有効である．

健常成人の1日に必要な鉄摂取量は男性が10 mg，女性は12 mgであるのに対し，日本人の1日の平均鉄摂取量は平均約8.3 mg（平成16年国民健康・栄養調査結果報告）である．熊本大学医学部附属病院の栄養士による鉄制限食の指導は，鉄分を多く含む食品（表1）を避け，1日の鉄摂取量は6 mgを目安に行っている．つまり日頃の

食事から比較すると約2/3程度に鉄量を抑えることが目標となる．これまで肝臓病によいとされてきた貝類（しじみ等）の摂取は，C型慢性肝炎の患者さんにはむしろ控えたほうがよいということになる．患者さんにとって鉄制限食は瀉血療法と違って自分で行うことができる治療法である．

4．瀉血療法による長期成績

Yanoらは，C型慢性肝炎患者に対して瀉血療法開始後，5年間の経過観察を行い，瀉血療法群ではコントロール群に対して血清ALT値の有意な改善とともに，組織学的に肝の線維化の進行が有意に抑制されたと報告した[7]．

またKatoらは，C型慢性肝炎患者に対して瀉血療法と鉄制限食の併用による除鉄療法を行い，6年間の経過観察を行った．その結果，除鉄療法開始前に比べて血清ALT値の有意な改善とともに，酸化ストレスによるDNA障害の指標でもあり，発癌にも密接に関与しているといわれている8-OHdG（8-hydroxydeoxyguanosine）の肝組織内の発現が，治療前の約10倍に増加していた状態から正常に復したことを報告した[8]．以上より除鉄療法により肝細胞癌の発癌抑制効果が得られるものと期待されている．

おわりに

C型慢性肝炎症例でC型肝炎ウイルス排除が無効な症例や困難な症例であり，かつ他の肝庇護療法では治療効果が不十分あるいは継続困難な症例に対して，瀉血療法は慢性肝炎の進行を抑制し，肝硬変，肝細胞癌への進展を阻止することが可能であり，鉄制限食の併用によりさらにその効果を高めることができる有効な治療法である．また近年NASH（非アルコール性脂肪性肝炎）においても鉄過剰沈着が報告されており，C型慢性肝炎にとどまらず鉄過剰状態を惹起する肝疾患全般にも臨床応用が可能な治療法と言える．

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Review

Does oxidative stress participate in the development of hepatocellular carcinoma?

YUTAKA SASAKI

Department of Gastroenterology and Hepatology, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan

Key words: oxidative stress, HCC, ROS, hepatitis virus, alcohol, NASH

Introduction

Hepatocellular carcinoma (HCC) ranks among the most common cancers in the world, and it is one of the leading causes of cancer death in Japan. Chronic hepatitis and liver cirrhosis associated with either hepatitis B virus (HBV) or hepatitis C virus (HCV) infection represent major risk factors for HCC development, being implicated in more than 80% of HCC cases worldwide.[1] Alcohol is also a strong primary cause when HCC develops in patients who are heavy alcoholics, most of whom have alcohol-induced cirrhosis. In addition, alcohol is a cofactor when associated with other causative factors including HCV, HBV, and diabetes mellitus.

On the other hand, oxidative stress can occur through overproduction of reactive oxygen species (ROS) or reactive nitrogen species (RNS) through either endogenous or exogenous insults, and is recognized to play an important role in the initiation and promotion of the events of carcinogenesis.[2,3] In this context, oxidative stress has emerged as a key player in the pathogenesis of chronic liver diseases and precancerous lesions, induced by HBV or HCV infection, because polymorphonuclear neutrophils (PMNs) in an inflamed liver are a major source of ROS.[4] Moreover, nonparenchymal cells, including Kupffer cells and macrophages, which release cytokines, are another cause of ROS induction in hepatocytes.[5] In addition, virus proteins may also generate oxidative stress. On the other hand, alcohol liver disease is associated with significant oxidative stress as well as iron accumulation.[6] The combined prooxidant potentials of ethanol and iron are at least additive and possibly synergistic with regard to induction of oxidative stress and antioxidant depletion in hepatocytes. Lately, calorie-enriched diets and lack of exercise are causing a worldwide surge in obesity, insulin resistance, and lipid accumulation in the liver (hepatic steatosis), which can lead to steatohepatitis. Evidence is accumulating that mitochondrial dysfunction plays a crucial role in the progression of nonalcoholic steatohepatitis (NASH).[7] Generation of ROS as well as RNS, accompanied by lipid peroxidation, further impair mitochondrial function.

From these points of view, oxidative stress plays a central role in the pathogenesis and progression of liver diseases. Important to carcinogenesis, unregulated or prolonged ROS production has been linked to mutation as well as to modification of gene expression. This review focuses on the mechanisms of ROS production and of ROS-induced cellular damage and modification of gene expression. Finally, this review discusses how ROS generated in chronic liver diseases participate in hepatocarcinogenesis.

Generation of ROS

ROS encompass a variety of partially reduced metabolites of oxygen (i.e., superoxide anions, hydrogen peroxide, and hydroxyl radicals), which possess higher reactivities than molecular oxygen and are generated endogenously as a consequence of normal cell functions or derived from external sources (Fig. 1).

Endogenous sources of ROS

Potential endogenous sources include oxidative phosphorylation, p450 metabolism, peroxisomes, and inflammatory cell activation.

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Reprint requests to: Y. Sasaki

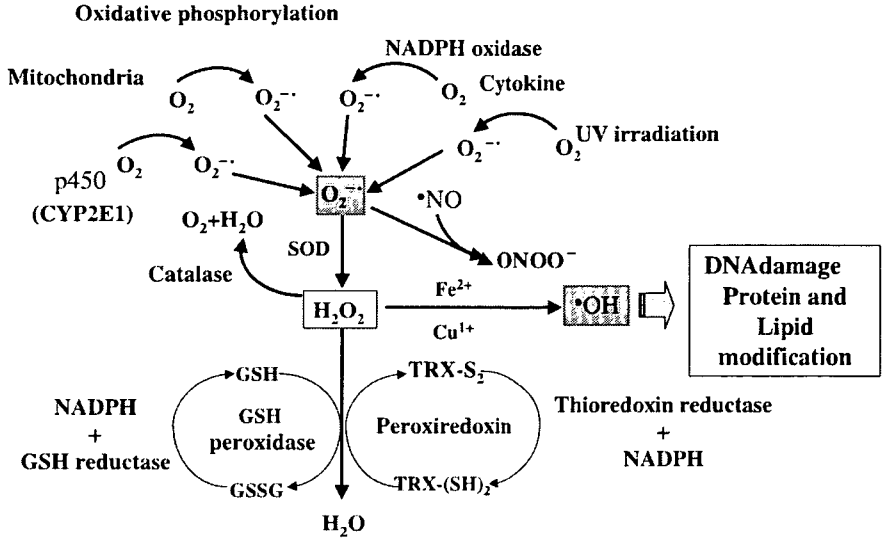

Fig. 1. Reactive oxygen species (ROS) and antioxidant defense mechanisms. ROS encompass a variety of partially reduced metabolites of oxygen possessing higher reactivities than molecular oxygen, and are generated endogenously as a consequence of normal cell functions or derived from external sources. A number of antioxidant defense systems have evolved to combat the accumulation of ROS. These include enzymatic and nonenzymatic molecules. *CYP2E1*, cytochrome p450 2E1; *SOD*, superoxide dismutase; *GSH*, reduced glutathione; *GSSG*, oxidized glutathione

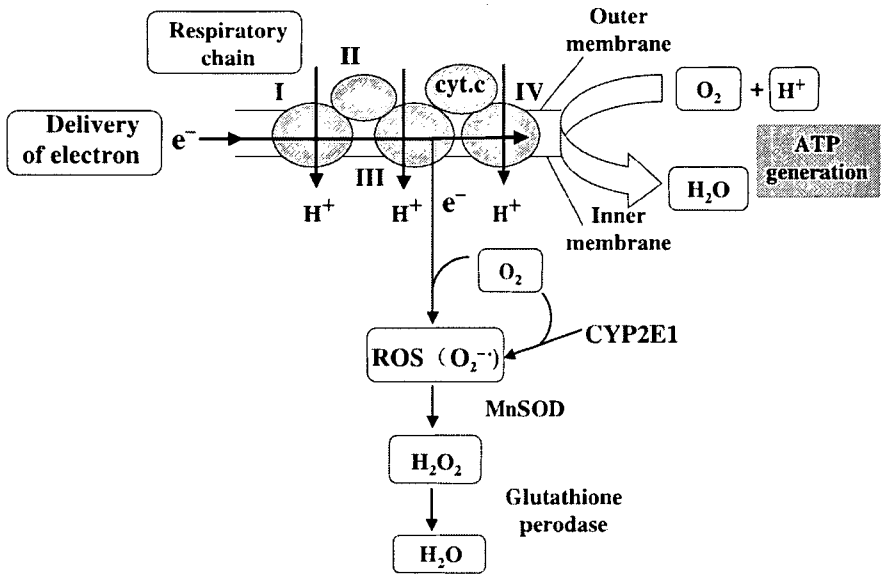

Fig. 2. Oxidative phosphorylation accompanied by ROS generation. During mitochondrial oxygen metabolism, most electrons provided to the respiratory chain migrate all the way along the respiratory chain and finally reach cytochrome c oxidase (complex IV). However, a fraction of these electrons can directly react with molecular oxygen to form ROS, primarily $O_2^{-\cdot}$. $O_2^{-\cdot}$ is, in turn, dismutated by mitochondrial MnSOD into H_2O_2, which is subsequently detoxified into H_2O by the mitochondrial glutathione peroxidase. *MnSOD*, manganese superoxide dismutase

During mitochondrial oxygen metabolism, most electrons provided to the respiratory chain migrate all the way along the respiratory chain, and finally reach cytochrome c oxidase (complex IV), where they safely combine with oxygen and protons to form H_2O. However, at several upstream sites along the respiratory chain, a fraction of these electrons can react directly with molecular oxygen to form ROS, primarily the superoxide anion ($O_2^{-\cdot}$). In other words, the majority of the oxygen is reduced to H_2O, but the approximately 4%–5% converted to $O_2^{-\cdot}$. $O_2^{-\cdot}$ is, in turn, dismutated by mitochondrial manganese superoxide dismutase (MnSOD) into hydrogen peroxide (H_2O_2), which is subsequently detoxified into H_2O by mitochondrial glutathione peroxidase[8] (Fig. 2). Glutathione peroxidase plays an important role in H_2O_2 detoxication, because mitochondria in hepatocytes do not have catalase. Of note, glutathione peroxidase needs an adequate amount of reduced glutathione (GSH) in order to detoxify H_2O_2. The depletion of mitochondrial GSH below a critical level may, therefore, lead to mitochondrial dysfunction and cell death.[9] In this way, even with healthy mitochondria, the respiratory chain generates ROS. Most ROS are detoxified into H_2O, and only a small amount of residual ROS persists. In contrast, damaged mitochondria generate larger amounts of ROS, which can alter mitochondria as well as other cellular components.

It should be emphasized that in the presence of reduced metal ions, especially iron and copper, H_2O_2 is subsequently converted through Fenton reactions ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\cdot}OH$) or a Harber-Weiss reaction

Table 1. Reactions involved in oxidant generation by neutrophils

Reactions	Products
NADPH oxidase $2O_2 + NADPH = 2O_2^{-\cdot} + NADP^+ + H^+$	$O_2^{-\cdot}$
SOD $2O_2^{-\cdot} + 2H^+ = O_2 + H_2O_2$	H_2O_2
MPO $Cl^- + H_2O_2 = HOCl + {}^{\cdot}OH$	·OH
Nitric oxide synthetase L-arg + O_2 + NADPH = ·NO + L-citrulline + $NADP^+$	·NO

SOD, superoxide dismutase; MPO, myeloperoxidase

into a hydroxyl radical (·OH). The hydroxyl radical (·OH) is highly reactive and can interact with nucleic acids, lipids, and proteins.[10]

In addition, metabolic activation and production of ROS by cytochrome p450 has been described.[11] ROS generation may vary considerably depending on the forms of p450: cytochrome p450 2E1 (CYP2E1) exhibits a higher rate of oxidative activity than the other forms of p450.[12] CYP2E1 is an important source of ROS in hepatocytes, not only in the cytosol and endoplasmic reticulum but also in mitochondria. CYP2E1 is involved in the microsomal ethanol oxidizing system (MEOS) and in the oxygenation of substrates such as ethanol, and can generate a prolonged burst of ROS at the site of substrate oxidation. Although most ethanol is oxidized by alcohol dehydrogenase (ADH), CYP2E1 assumes a more important role in ethanol oxidation at elevated levels of ethanol or after chronic consumption of ethanol. Moreover, CYP2E1 has the ability to metabolize and activate many other toxicologically substrates, including acetaminophen and *N*-nitrosodimethylamine, to more toxic products.[13] Thus, mitochondrial CYP2E1 can produce ROS and induce lipid peroxidation.

Production of ROS derived from peroxisomes also has been proposed. Compounds such as peroxisome proliferators are potent inducers of p450 4A and induce formation of peroxisomes, accompanied by an increase in H_2O_2 production. Consequently, H_2O_2 escapes from peroxisomes and shifts the cellular redox balance toward an oxidative state.[14]

On the other hand, neutrophils and nonparenchymal cells are additional endogenous sources of ROS. An association between chronic inflammation and the development of cancer has been recognized for a long time.[4] Inflammatory neutrophils are a major source of oxidants in an inflamed liver, and release of ROS from these cells provides a plausible mechanism by which chronic hepatitis and HCC development might be related. In the course of their defense activities, neutrophils produce a vast amount of oxidants. The whole spectrum of oxidants generated by neutrophils is due to the actions of four different enzymes (Table 1). Among these enzymes, NADPH oxidase initiates oxidant generation. Superoxide anion ($O_2^{-\cdot}$), generated by NADPH oxidase, is a substrate for the enzyme superoxide dismutase (SOD), which catalyzes the formation of H_2O_2 from $O_2^{-\cdot}$ (Fig. 1). H_2O_2 is relatively stable and capable of diffusing and penetrating the cellular membrane, which provides to neutrophils the possibility of action at a distance. However, most H_2O_2 is consumed by myeloperoxidase (MPO). MPO is the most abundant enzyme in neutrophils and catalyzes the conversion of H_2O_2 into HOCl. In addition, neutrophils are also able to produce RNS, the production of which is facilitated by inducible nitric oxide synthase (iNOS), which catalyzes the production of ·NO from oxygen, L-arginine, and NADPH. The ability of neutrophils to yield ·NO is much less than that of macrophages. In any case, the four enzymes in neutrophils generate four types of oxidants: $O_2^{-\cdot}$, H_2O_2, HOCl, and ·NO. These products constantly interact with one another, causing the formation of a myriad of oxidants, among which ·OH is the most DNA-reactive compound.[5] Furthermore, the release of biologically active molecules, such as tumor necrosis factor (TNF)-α and ROS, from activated Kupffer cells has been implicated in hepatocarcinogenesis.

In this regard, activation of Kupffer cells directly or indirectly by toxic agents results in the release of an array of inflammatory mediators, growth factors, and ROS. The activation of Kupffer cells appears to modu-

late acute hepatocyte injury as well as chronic liver responses, including hepatocarcinogenesis.[15]

Exogenous sources of ROS

ROS can be generated exogenously. Environmental agents, including xenobiotics, radiation, metal ions, and some peroxisome-proliferating compounds, are among the classes of compounds that have been shown to induce ROS and damage in vitro and in vivo. 2-butoxyethanol is an example of a chemical that produces ROS indirectly, resulting in liver cancer in mice.[16]

Antioxidant defense mechanisms

A number of antioxidant defense systems have evolved to combat the accumulation of ROS (Fig. 1). These include enzymatic molecules (e.g., SOD, catalase, and glutathione peroxidase), and nonenzymatic molecules (e.g., glutathione, vitamins C and E, coenzyme Q, flavonoids). SODs are localized to the cytosol and mitochondria, and reduce $O_2^{-\cdot}$ to H_2O_2 and H_2O. Glutathione peroxidases, which are also localized to the cytosol and mitochondria, remove the majority of H_2O_2,[17] while catalase, located in peroxisomes, contributes to the removal of yet more H_2O_2.

Among the molecules constituting antioxidant defense systems, GSH redox cycle and redox-sensitive proteins, including glutathione and thioredoxin, are of primary importance. Of note, the intracellular redox state is determined by the relative ratio of the reduced and oxidized forms of each redox pair. In this regard, glutathione is the most important nonprotein thiol to the overall redox balance, because the intracellular concentration of glutathione is 500- to 1000-fold higher than the other redox-regulatory molecules. Glutathione is present in reduced (GSH) and oxidized (GSSG) forms. The concentration of GSH is 10- to 100-fold higher; therefore, GSH prevails over GSSG. An increase in the GSSG level can arise from the breakdown of H_2O_2 by GSH peroxidase. Because the concentration of GSSG is relatively low, the oxidation of a limited amount of GSH to GSSG can dramatically change this ratio, affecting the cellular redox state. GSSG can be reduced to GSH by NADPH-dependent glutathione reductase. Thus, the GSH/GSSG ratio is strictly regulated, and can be maintained even in the face of oxidative stress by increasing GSH reductase activity or elimination of GSSG.[18] As an antioxidant, glutathione metabolizes ROS primarily by serving as a cofactor for GSH-dependent enzymes such as GSH peroxidase, and also modulates the activity of thiol-dependent enzymes that contain cysteine residues sensitive to redox change.[19]

The cellular redox state is regulated by cellular thiols, including GSH and thioredoxin (TRX)-1. There is considerable evidence that the TRX system is as important as the GSH system in cellular redox regulation against oxidative stress. TRX, a family of small proteins that contain a conserved redox active center, is known to protect cells against ROS.[20] TRX-1 has a variety of biological activities, including the scavenging of ROS and the regulation of redox-sensitive molecules such as NFκB. Recent studies indicate that TRX-1 is induced to protect host cells from various types of stresses, including ROS, viral infection, and ischemic insult.[21] Moreover, serum TRX-1 levels are recognized as an oxidative stress marker.[22]

Oxidative stress and cellular responses

ROS generation often exceeds a cell's antioxidant capacity, leading to a condition called oxidative stress. Oxidative stress can occur through overproduction of ROS and RNS through endogenous and exogenous insults, and interacts with a wide range of intracellular molecules, eliciting cytostatic/cytotoxic damage to cellular DNA, proteins, and lipids. Consequently, oxidative stress is implicated in a wide variety of disease processes, including atherosclerosis, diabetes mellitus, and pulmonary fibrosis, and is considered to be a major factor of aging.

Under normal metabolic processes, it is estimated that as many as 10000 oxidative hits take place per cell per day. Oxidative hits would increase substantially following bursts of cellular metabolism, inflammation, or depletion of cellular antioxidants.[23]

Nuclear DNA damage

Among many forms of ROS, $^{\cdot}OH$, in particular has been shown to generate a number of oxidized DNA lesions. Because the migration of $^{\cdot}OH$ is limited, it reacts rapidly with cellular components. H_2O_2, a precursor to $^{\cdot}OH$, is less reactive but more readily diffusible and thus more likely to be involved in the formation of oxidized bases. Recent attention has been focused on reactive oxygen products formed in DNA, with particular emphasis on the formation and repair of 8-hydroxy deoxyguanosine (8-OHdG), one of the important lesions in base mispairing. There are two mechanisms of the formation of 8-OHdG in cellular DNA: direct interaction of ROS with guanine at the C8 position, and guanine oxidization in the nucleotide pool during DNA replication.[24–26] In normal cells, it has been estimated that around 200 8-OHdG genomic DNA lesions are formed per cell per day by the two mechanisms described above. These oxidative DNA lesions result in site-specific

mutagenesis and produce G to T transversions that are widely found in mutated oncogenes and tumor suppressor genes.[27] In addition, during DNA replication, 8-OHdG in the nucleotide pool can be incorporated into DNA, resulting in A:T to C:G transversions. Further support for the involvement of 8-OHdG in carcinogenesis comes from studies showing that 8-OHdG produces dose-related increases in cellular transformation, which can be prevented by antioxidants.[28] Conversely, DNA repair enzymes and a repair system exist and function to remove altered bases produced by oxidative stress. 8-OHdG lesions in cellular DNA are repaired by the action of formamidopyrimidine-DNA-glycosylase (FPG), a product of the human *MMH* gene.[29] In nuclear DNA, around 90% of oxidized bases are repaired by single-nucleotide repair mechanisms, and the remaining 10% by long-patch base excision repair, indicating that single-nucleotide repair is the primary pathway for the repair of 8-OHdG.

DNA methylation

DNA methylation is an important regulator of gene expression, decreased methylation being associated with increased gene expression. In this context, many cancer cells have been shown to exhibit global hypomethylation of DNA compared with control cells. In particular, hypomethylation of tumor-promoting genes has been proposed as a possible mechanism for cancer development. Oncogenes can become hypomethylated and their expression amplified. In contrast, the promoters of some tumor suppressor genes are methylated, resulting in their inactivation.[30,31] Hypermethylation of genes may inhibit transcription of tumor suppressor genes. Although mutation in the coding region of genes has been considered the major mechanism of inactivation of the tumor suppressor genes, aberrant DNA methylation of the CpG islands in the promoter region has recently emerged as an alternative mechanism for the silencing of tumor suppressor genes, and may be one of the earliest events in the neoplastic transformation of cells.[32,33]

Among the agents and situations that can alter methylation status, ROS are most potent and can modify DNA methylation. In particular, oxidative DNA damage elicited by ROS can result in decreased DNA methylation.[34] In this regard, the formation of 8-OHdG in DNA can lead to hypomethylation, because the presence of 8-OHdG in CpG islands inhibits the methylation of adjacent C residues by methyltransferase. Additionally, 8-OHdG formation can interfere with the normal function of DNA methyltransferase and alter DNA methylation.

With regard to HCC development, hypermethylation of CpG islands in the $p16^{INK4}$ promoter region has been detected in not only HCC but also liver cirrhosis and chronic hepatitis.[35] In addition, hypermethylation has also been reported on CpG islands in the 5′ noncoding region of the silencing of the suppressor cytokine signaling-1 (*SOCS-1*) gene.[36] The precise mechanisms leading to hypermethylation of CpG islands are not known. However, in nickel carcinogenesis, hypermethylation of $p16^{INK4a}$ is apparently induced by ROS-mediated mitogen-activated protein (MAP) kinase activation.[37] Although it has been reported that levels of methyltransferase mRNA are increased in liver tissue in chronic hepatitis and liver cirrhosis compared with in normal liver tissue,[38] several lines of evidence suggest that there is no correlation between DNA methyltransferase activity and aberrant methylation.[39] Other mechanisms such as loss of protection against de novo methylation may account for aberrant methylation of tumor suppressor genes.[33]

Target genes exhibiting a significantly higher frequency of changes in DNA methylation in tumor tissues than in the neighboring noncancerous tissues have been reported to represent a late phase of carcinogenesis, with early-phase specific changes occurring at the same frequency in both cancerous and noncancerous tissues.[40] In any case, hypomethylation of tumor promoting genes (oncogenes) and hypermethylation of tumor suppressor genes contribute to hepatocarcinogenesis, and their detection is promising in the follow-up of patients at a high risk of developing HCC.

Mitochondrial DNA damage

Mitochondrial DNA (mtDNA) is a circular double-stranded molecule located in the mitochondrial matrix, and it is extremely sensitive to oxidative stress owing to (1) its proximity to the inner membrane, where ROS is mainly generated; (2) the absence of protective histone; and (3) incomplete repair mechanisms in the mitochondria. These findings may account for the increased frequency of mtDNA mutations seen in tumor cells. Indeed, the mutation rate in mtDNA is at least two orders of magnitude higher than that in nuclear DNA. Mutations in mtDNA encoding oxidative phosphorylation would disturb the respiratory chain associated with an increase in ROS, and affect cellular ATP levels, resulting in the prevention of cell cycle progression. Furthermore, it has been shown that fragments of mtDNA are inserted into nuclear DNA, which has been suggested as a mechanism for activation of oncogenes.[41] In this regard, mtDNA mutations have been identified in a variety of tumors.

Thus, mtDNA damage may constitute one step in carcinogenesis via mitochondria-derived ROS and the insertion of mitochondrial genes into nuclear DNA.

Lipid peroxidation

Free radical-mediated damage to cellular membranes results in lipid peroxidation, a process that generates a variety of DNA-reactive aldehydes, such as 4-hydroxy-2-nonenal (4HNE), and malondialdehyde (MDA). These products have cellular half-lives upward of 2–3 min and therefore are capable of diffusing from their site of production to more distant sites within the cell to interact with DNA or proteins. In particular, 4HNE can react with DNA, yielding etheno (epsilon)-modified DNA bases.[42] Hepatic etheno-adduct levels are significantly higher in patients with alcohol-related hepatitis, fatty liver, fibrosis, or cirrhosis than in those with asymptomatic livers, indicating that etheno-adducts may serve as potential markers for assessing progression of inflammatory cancer-prone diseases.

In this way, the diffusible and electrophilic characteristics of lipid peroxidative products may contribute much to carcinogenesis.

Signal transduction pathways

At the cellular level, oxidative stress elicits a variety of cellular responses, ranging from proliferation to growth arrest, senescence, and cell death. Effects within cells appear to be cell-specific and dependent on the form and intercellular concentration of ROS.

ROS function to induce cell proliferation during the tumor progression stage of carcinogenesis. Both H_2O_2 and $O_2^{-\cdot}$ induce mitogenesis and cell proliferation in several cell lines. In addition, a reduction in cellular oxidants by antioxidants inhibits cell proliferation. In contrast, high concentrations of ROS trigger apoptotic signaling pathways, resulting in cell death.[43] Whatever effect is observed, it largely reflects the balance among a variety of intracellular stress signalings activated in response to the oxidative stress.[44]

Mitogen-activated protein kinases (MAPKs) comprise a large number of serine/threonine kinases involved in regulating a wide array of cellular processes, including proliferation, differentiation, and apoptosis. On the basis of structural differences, they are divided into three multimember subfamilies: extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38kinases (p38MAPK). The latter two are categorized as stress-activated protein kinases (SAPKs).

The ERK pathway is most linked to the regulation of cell proliferation, while the SAPKs (JNK and p38MAPK) pathways are more strongly tied to stress. It is clear that oxidative stress leads to substantial activation of ERK. Two possible mechanisms have been proposed for this effect. One is that oxidative stress may mimic the effects of the ligand–receptor interaction through the modification of cysteine residues on the receptor.[45] The other is that oxidative stress may inactivate GSH-sensitive phosphatases necessary for dephosphorylation of the receptor.[46] However, other experiments have indicated that ERK activation can contribute to apoptosis in response to oxidative stress. What determines whether ERK will act in a proapoptotic or antiapoptotic fashion remains to be clarified, but the kinetics and durations of its activation may be important factors. Specifically, if activation of ERK occurs rapidly and is more transient, ERK activity may enhance survival, whereas if activation tends to be delayed and sustained, ERK activity may induce apoptosis.

The SAPK (JNK and p38MAPK) pathways are noted for their activation by a wide range of stresses. For oxidative stress-induced activation of these pathways, change in the cellular redox state seems to be a key factor. Under normal conditions, the redox regulatory protein TRX has been shown to bind and inhibit apoptosis signal-regulating kinase (ASK1), a MAPK kinase kinase (MAPKKK) involved in both JNK and p38MAPK activation.[47] However, oxidative stress causes dissociation of the TRX-ASK1 complex, leading to activation of JNK and p38MAPK. As is the case with TRX, under nonstress conditions, GST binds to JNK and inhibits its activity, but this interaction is disrupted by oxidative stress.[48]

Thus, oxidative stress may act at multiple levels in the SAPK pathways to regulate their activities. The influence of JNK activation on cell survival following oxidative stress is complex and controversial. Many studies have shown that JNK activation is correlated with cell death or apoptosis. The role of p38MAPK is also controversial. Previous studies have yielded evidence for proapoptotic[49] as well as antiapoptotic[50] activity. These signaling pathways exert their phenotypic influences through modulation of transcriptional factor activities.

Gene expression

The most significant effects of oxidative stress on signaling pathways have been observed in the MAPK/AP-1 and NFκB pathways.[51] Activation of these transcriptional factors is involved in both cell proliferation and apoptosis. The cellular redox state appears to influence the selective activation of these transcriptional factors and therefore, may help explain the observation that either cell death or cell proliferation may result from exposure to oxidative stress.

AP-1 is a collection of dimeric basic region leucine zipper (bZIP) proteins, including the Jun (c-JunM, JunB, JunD), Fos (FosB, Fra-1, Fra-2), and ATF families.[52] A common effect of AP-1 activation is an increase in cell proliferation. One of the genes regulated by AP-1

is *cyclinD1*, which supports the inference that AP-1 promotes entry into the cell division cycle.[53] AP-1 proteins also function as positive or negative regulators of apoptosis. Whether AP-1 induces or inhibits apoptosis is dependent on the balance between proapoptotic and antiapoptotic target genes, which varies from one cell type to another, and the durations of stimuli. Finally, AP-1 proteins participate in oncogenic transformation through interaction with activated oncogenes.[54] Among these oncogenes, *c-Jun* has been the most studied in this regard. Just as with its upstream regulator JNK, both pro- and antiapoptotic functions have been ascribed to c-Jun. Like JNK, c-Jun functions in a manner that is cell-type specific, agent specific, or both.

The NFκB family of transcriptional factors is composed of homodimers or heterodimers of Rel proteins consisting of p50 (NFκB1), p52 (NFκB2), and so on.[55] The predominant mechanism by which NFκB is activated by various stimuli is through the phosphorylation of IκB. IκB is an inhibitory protein that under normal conditions binds to NFκB, preventing its access to DNA. However, the phosphorylation of IκB results in its ubiquitination and degradation, freeing NFκB to translocate to the nucleus and activate transcription. A number of different kinases, including Iκ-kinase (IKK), and NFκB-inducing kinase (NIK), have been reported to phosphorylate IκB.[56] Many of these kinases offer obvious points for cross-talk with the signaling pathways known to be activated by ROS. Virtually, every step of the NFκB signaling cascade consists of redox-sensitive proteins whose activities are modulated upon changes in ROS.[57] In addition, NFκB needs to be in reduced form to exhibit DNA-binding activity. Ergo, reducing agents enhance DNA activity of NFκB, while oxidizing agents inhibit this activity. Activation of NFκB has been considered to be linked to carcinogenesis, because NFκB regulates several genes involved in cell transformation, proliferation, angiogenesis, and cell survival.[58] In this context, a large number of NFκB target genes have antiapoptotic functions. These include those coding for TNF-α, TNF receptor-associated factor1 (TRAF1), TRAF5, and cellular inhibitors of apoptosis proteins (CIAPs).[59] NFκB is also involved in regulating the expression of *Bcl-Xl*, an antiapoptotic member of the Bcl-2 family. Accordingly, NFκB expression has been shown to exert protective effects under various conditions. Carcinogens and tumor promoters, including UV radiation, phorbol esters, alcohol, and benzo(a)pyrene, are among the external stimuli that activate NFκB.

The tumor suppressor protein p53 exerts its action in response to oxidative stress. Indeed, many chemotherapeutic agents generate ROS, leading to activation of p53. Activation of p53 by oxidative stress can result in either growth arrest or apoptosis. What determines this decision is unclear, but a number of factors are involved, including the cell type and the magnitude and severity of the damage. Oxidative stress contributes to p53 activation in many ways; both JNK and p38MAPK can phosphorylate p53, and both have been implicated in regulating p53 expression levels through stabilization of the p53 protein. Downstream targets of p53 activation have been identified. Genes linked to growth arrest include *p21/Waf1*, *GADD45*, and *14-3-3*, which are important in mediating G2/M arrest,[60] while genes linked to apoptosis include *Bax*, a proapoptotic Bcl-2 family member, and *Fas*. On the other hand, elimination of p53 function enhances survival, even if the cells are exposed to oxidative stress. This is likely to be an important factor contributing to the chemotherapeutic resistance of cancer in which the *p53* gene is mutated. In this regard, allelic loss on chromosome 17p is among the most common genetic abnormalities associated with the genesis of many cancers, including HCCs. Loss of p53 function occurs mainly through allelic loss at chromosome17p13, where the *p53* gene is located.[61] Loss of heterozygosity at chromosome 17p13 has been reported in 25%–60% of HCCs, and the worldwide prevalence of *p53* mutation is around 28%.[62] These findings indicate that preserved p53 function is a requisite for a chemotherapeutic approach against HCC.

Of note, p53 activation itself results in the generation of ROS. Although the exact mechanism whereby p53 activation leads to increase in oxidative stress remains unclear, it is possible that p53 activation modulates expression of the genes involved in regulating the cellular redox state.[63] p53 activation can also interfere with survival signals to render cells permissive to apoptosis; p53 represses expression of *Bcl-2*, an antiapoptotic gene of the Bcl-2 family, and increases expression of *Bax*, a proapoptotic gene of the Bcl-2 family.[64] Thus, an important consequence of ROS-induced p53 activation is a further increase in the cellular level of oxidative stress.

Through regulation of transcriptional factors and disruption of signal pathways, ROS are involved in the maintenance of concerted networks of gene expression that may correlate with neoplastic development.

Viral infection-associated oxidative stress and hepatocarcinogenesis

HCV infection-related oxidative stress

HCV infection frequently leads to severe liver diseases, including liver cirrhosis and HCC. Although the precise pathogenesis of chronic liver disease remains obscure, oxidative stress has been focused on as a central player in the progression of many pathological conditions in HCV-related liver diseases. Indeed, HCV infection is

characterized by increased markers of oxidative stress: lipid peroxidation and oxidative DNA damage are enhanced in serum and liver specimens of patients with HCV infection.[65,66] The increased oxidative stress in HCV infection may be explained by chronic inflammation, and the continued generation of ROS and RNS in the liver may be accounted for by NAD(P)H oxidase, especially Nox-2 in PMNs and Kupffer cells in the liver.[67] It has become evident that structural and nonstructural proteins of HCV are involved in the generation of ROS in an infected liver. For instance, NS3 protein of HCV has been found to activate Nox-2 proteins of Kupffer cells to induce apoptosis of T cells, NK cells, and NKT cells.[68] Nox2 protein increases generation of ROS and other reactive species, which can exert oxidative stress on nearby cells. Furthermore, HCV can directly induce oxidative stress in hepatocytes. HCV core protein has been associated with increased ROS, decreased intracellular and/or mitochondrial GSH content, and increased levels of lipid peroxidation products, leading to hepatocarcinogenesis.[69] A recent study further showed increased oxidation of mitochondrial GSH and decreased NADPH content in liver mitochondria from transgenic mice expressing the HCV structural proteins, including core protein. Furthermore, there was reduced activity of the electron transport complex I and increased generation of ROS from complex I substrates. Incubation of control mitochondria in vivo with recombinant core protein also causes oxidation of GSH, complex I inhibition, and increased ROS production. In addition, HCV core protein enhances mitochondrial Ca^{2+} uptake, resulting in inhibition of electron transport accompanied by ROS production at complex I.[70] These findings are consistent with the mitochondrial abnormalities found in vivo in the core-expressing animal model as well as in patients with HCV infection.[71]

It has been shown that HCV also induces ER (endoplasmic reticulum) stress.[72,73] For instance, HCV NS5A protein perturbs the host redox state. NS5A induces an accumulation of misfolded proteins and generation of ER stress with the subsequent release of Ca^{2+} from the ER, followed by mitochondrial Ca^{2+} uptake and the generation of ROS in the mitochondria. ER stress is a homeostatic mechanism that regulates cellular metabolism and protein synthesis in response to perturbations in protein folding and biosynthesis.[74] It has been hypothesized that persistent ER stress induction may result in intracellular and extracellular accumulation of DNA-damaging factors that could predispose a cell to mutagenesis.

It should be noted that the cellular redox environment is tightly regulated by antioxidant/reductants as well as antioxidant enzymes. During oxidative stress, many of the antioxidant enzymes are upregulated in response to oxidative stress. It is interesting to note that core protein, but not NS5A, decreases the GSH content. Similarly, no compensatory induction of heme oxygenase 1 (HO-1) or catalase was detected, despite ROS production in the cells expressing core protein. In contrast, NS5A can increase MnSOD, HO-1, and GSH.[75] These findings indicate that HCV may not only increase ROS production but also modulate antioxidant genes.

Recent and striking evidence of the causal role of HCV in HCC development derives from studies using transgenic mice. Transgenic mouse expressing HCV core protein shows an increased accumulation of ROS, which correlates with HCC development,[76] and transient expression of NS5A alters intracellular calcium levels, leading to oxidative stress and activity of STAT3 and NFκB.[77] Oxidative DNA damage increases chromosomal aberrations associated with cell transformation, which may account, in part, for the evidence implicating oxidative stress in the development of HCV-associated HCC.[78]

As described before, ROS may promote pathogenesis through cell signaling pathways. NS5A-induced oxidative stress has been revealed to activate NFκB, leading to further activation of the *COX-2* gene.[79] Activation of COX-2, in turn, would result in increased PGE2 production, which can inhibit apoptosis of the tumor cells, induce proliferation, and promote metastasis.[80] Oxidative stress also activates the MAPKs, which have profound effects on cell growth and may promote transformation. Indeed, through ROS generation, HCV infection induces activation of ERK, a conventional MAPK, in human HCC tissue.[81]

On the other hand, iron overload, which is often observed in HCV-infected hepatocytes, may also participate in liver injury.[82] Moreover, iron overload induces mitochondrial injury and increases the risk of HCC development in transgenic mice expressing the HCV polyprotein.[83] In contrast, iron reduction therapy by repeated phlebotomy improves hepatocyte injury in patients with HCV infection.[84]

Furthermore, steatosis is another common feature in HCV-infected hepatocytes.[71] In this regard, high β oxidation rates increase electron delivery to the mitochondrial respiratory chain, leading to an imbalance between a high input and a restricted flow of electrons. Consequently, overproduction of complex I and III in the respiratory chain may lead to their reaction with oxygen to form ROS. In this way, steatosis in hepatocytes of patients with HCV infection may contribute, in part, to hepatocarcinogenesis, through mitochondrial dysfunction and ROS generation.

Taken together, these facts suggest that ROS production associated with HCV infection may cooperate with other factors and promote hepatocarcinogenesis.

HBV-related oxidative stress

HBV is one of the causative agents of acute and chronic hepatitis, cirrhosis, and HCC. Transgenic mice expressing the HBV large envelope protein or surface antigen (HBsAg), display the generation of oxidative stress and DNA damage, leading to development of HCCs.[85,86] In addition, HBV X (HBx) protein has drawn considerable attention owing to its role in viral replication and the generation of HCC. Although the oncogenic property of HBx remains controversial, it has been established that HBx protein contributes to HCC development, in conjunction with genotoxic stresses and/or oncogene activation.[87,88] On the other hand, HBx protein binds to a voltage-dependent anion channel (VDAC3) and alters the mitochondrial transmembrane potential, leading to enhanced ROS generation. Consequently, the association of HBx protein with mitochondria induces the activation of transcriptional factors, including STAT 3 and NFκB, which is prevented by antioxidants or overexpression of MnSOD.[89] These observations indicate that HBx protein participates in the development of HCC via ROS generation.

Alcohol-associated oxidative stress and HCCs

There is compelling evidence that chronic alcohol consumption increases the risk of developing HCC. However, the exact role of alcohol in the development of HCC, compared with chronic HBV or HCV infection, is still incompletely defined, since ethanol itself is not a carcinogen.[90] Chronic alcohol consumption leads to multiple biochemical changes that could directly initiate or potentiate liver cancer. Involvement of ROS generated by ethanol consumption is the focus of this section.

Nongenotoxic mechanisms

Evidence has accumulated that acetaldehyde, the main metabolite of ethanol, is predominantly responsible for alcohol-associated carcinogenesis, because acetaldehyde is carcinogenic and mutagenic, binds to DNA or protein, destroys folate, and results in secondary hyperregeneration. In this context, it has become evident that the binding to DNA to form stable adducts represents one mechanism whereby acetaldehyde can trigger the occurrence of replication errors or mutations in oncogenes or tumor suppressor genes.[91] Furthermore, acetaldehyde inhibits methylguanyltransferase, an enzyme important for the repair of adducts by alkylating agents.[92] Moreover, acetaldehyde elicits overproduction of ROS through aldehyde metabolism in mitochondria, which induces hepatocyte apoptosis,[93] and increases ROS formation in hepatic stellate cells, leading to activation of stellate cells and promotion of fibrosis.[94]

Both generation and degradation of acetaldehyde is modulated by polymorphisms or mutations of the genes responsible for the enzyme involved. In Japan and other Asian countries, a high percentage of individuals carry a mutation of the aldehyde dehydrogenase 2 (*ALDH2*) gene. In humans, there are at least four classes of ALDH isozymes, and mitochondrial class 2 ALDH (ALDH2) is primarily responsible for acetaldehyde oxidation. The gene encoding ALDH2 enzyme is polymorphic with two distinct alleles: *ALDH2*1* and *ALDH2*2*. *ALDH2*2* results from a single point mutation in chromosome 6 coding the normal *ALDH2*1*. While individuals homozygous for the mutated *ALDH2*2* are completely devoid of ALDH2 activity, heterozygous individuals show 30%–50% of ALDH2 activity. Blood acetaldehyde levels of *ALDH2*2* homozygous individuals are 6–20 times higher than those of *ALDH2*1* individuals. In this regard, Japanese studies have shown that the odds ratio for HCC is higher in patients with HCV infection and alcohol consumption who lack aldehyde dehydrogenase activity (*ALDH2*2* homozygous) than in patients with HCV infection and alcohol consumption who have aldehyde dehydrogenase activity (*ALDH2*1* homozygous or *ALDH2*1/2* heterozygous). In addition to mutation of the *ALDH2* gene, polymorphisms of alcohol dehydrogenase (ADH) 1B may also modulate acetaldehyde levels; the *ADH1B*2* allele encodes for an enzyme that is around 40 times more active than the enzyme encoded by the *ADH1B*1* allele. In Japan and other Asian countries, *ADH1B*2* allele frequency is high, leading to generation of high amounts of acetaldehyde.[95]

Thus, polymorphisms or mutations of the genes responsible for the enzyme involved in acetaldehyde metabolism may modulate the role of acetaldehyde in hepatocarcinogenesis.[96]

On the other hand, CYP2E1 is involved in the MEOS and is induced by heavy ethanol use. The concentration of CYP2E1 in the liver can be correlated with the generation of α-hydroxyethyl radicals and with lipid peroxidation. Induction of CYP2E1 may contribute to the development of HCC by two distinct mechanisms. First, CYP2E1 generates ROS, leading to apoptosis or DNA damage and cancer initiation.[97] Second, CYP2E1 oxidizes xenobiotics, including the procarcinogens (nitrosamines, aflatoxin, vinylchloride, etc), causing them to become carcinogens.[98] The *CYP2E1* gene is polymorphic in the promoter region, and the relatively rare *c2* allele is associated with increased *CYP2E1* gene expression. Increased frequency of the *c2* allele has been reported in HCC patients who have a history of alcohol intake, whereas the frequency of the *c2* allele

in HCC patients who do not drink is similar to that of controls.[99]

In this way, genetic differences, especially in the aldehyde dehydrogenase and *CYP2E1* genes, may increase the risk of the HCC development in the people who drink alcohol.

Alcoholic liver disease is associated with significant oxidative stress as well as the hepatic accumulation of iron, which also initiates oxidative stress. It is widely recognized that hepatic iron overload develops in a significant portion of individuals who consume alcohol on a chronic basis. The role of iron, alone or in combination with ethanol, has been difficult to establish, because this transition metal is among the most abundant elements and is essential for healthy individuals. It has been established that iron augments ROS-induced damage by catalyzing the formation of hydroxyl radicals. On the other hand, production of ethanol-derived α-hydroxyethyl radical ($CHOHCH_3$) has been observed in the livers of animals chronically treated with ethanol.[100] This radical has been proposed to mediate its cytotoxic effects through adduct formation with cellular proteins. Therefore, the unregulated and sustained generation of such free hydroxyl radicals and ethanol-derived α-hydroxyethyl radicals undoubtedly contributes to the cocarcinogenic actions of ethanol and iron. On the other hand, Kupffer cells play an important role in cellular responses associated with inflammation and cell death. Of particular importance is the observation that elevated nonheme iron concentrations in Kupffer cells isolated from alcohol-treated rats are associated with increased NFκB binding as well as increased mRNA for TNF-α.[101] This observation has significant implications with regard to the role of iron as a potential signaling molecule in modulating hepatic TNF-α. In this regard, TNF-α is released by Kupffer cells stimulated by lipopolysaccharide (LPS). In addition to its proinflammatory property, initiation of TNF-α-mediated hepatotoxicity has been linked to the action of LPS[102] and oxidative stress by ethanol.[103] Thus, accumulation of iron in Kupffer cells is a key event for activation or sensitization of these cells to stimuli such as LPS or other extracellular effectors.

Alteration of DNA methylation

ROS are most important and can modify DNA methylation, and, in particular, oxidative DNA damage can result in decreased DNA methylation.[104] Thus, hypomethylation is a plausible consequence of the metabolic alterations associated with ethanol consumption. The generation of ROS by ethanol consumption diminishes the activity of methyladenosyltransferase II in chronic alcohol cirrhosis, accompanied by decreased levels of *S*-adenosylmethionine, the methyl donor for DNA methylation. In addition, ethanol increases homocysteine levels, followed by an increase in *S*-adenosylhomocysteine levels.[105] Chronic ethanol consumption also decreases glutathione levels in the liver, and enhances the susceptibility of the liver to alcohol-related oxidative stress. Furthermore, chronic ethanol consumption interacts with the absorption and subsequent metabolism of vitamin B, which is involved in hepatic transmethylation reactions, resulting in impaired methyl group synthesis and transfer.[105] To date, it is evident that dietary depletion of methionine, choline, and folate leads to DNA hypomethylation, in particular, hypomethylation of oncogenes, including *c-Ha-ras*, *c-Ki-ras*, and *c-fos*, and to DNA strand breaks, resulting in an increase in the incidence of HCC.[106]

Thus, several mechanisms, including ROS generation and dietary depletion, have been proposed by which ethanol can interact with DNA methylation and thereby enhance carcinogenesis.

Nonalcoholic fatty liver disease-associated oxidative stress and HCCs

Nonalcoholic fatty liver disease (NAFLD) is now recognized as one of the most common causes of liver disease in the United States and other countries. NAFLD ranges from simple fatty liver to nonalcoholic steatohepatitis (NASH), which may lead to cryptogenic cirrhosis and, in some cases, to HCC. The marked apoptosis rate in patients with NASH requires a compensatory increase in the cell proliferation rate of progenitor cells to maintain liver mass.[107] Concomitantly, both ROS and lipid peroxidation products damage DNA. The combination of DNA damage and increased cell proliferation causes gene mutations. As these mutations accumulate over the years, accompanied by constant apoptotic pressure, cells that resist apoptosis or escape the control of the cell cycle may be selected for, finally allowing the development of HCC. However, although some HCC patients exhibit NASH, whether HCC is part of the natural history of NASH has been controversial. In this regard, no case of HCC was found during 5 years of follow-up in one prospective study, in striking contrast to 1%–3% per year with hepatitis C infection.[108] It needs to be clarified whether metabolic determinants, such as steatosis, insulin resistance, and glucose intolerance, predispose a person to liver cancer, allowing the determination as to whether fibrotic NASH is a cause of HCC, or whether the metabolic factors serve as epigenetic determinants of hepatocarcinogenesis initiated by other causative factors.