コラーゲン免疫14日後に脾細胞を分離し*in vitro*でタイプIIコラーゲンにて再刺激後の培養上清中のサイトカインを測定すると，DBA/J1-ICOS$^{-/-}$F6ではDBA/J1-ICOS$^{+/+}$F6と比較してIFNγ，TNFαの産生は同等であったが，IL-17の産生が有意に低下していた．一方，DBA/J1-ICOS$^{+/+}$F6のnaive Th細胞を抗CD3抗体単独で刺激するとIL-17産生が誘導された．このIL-17産生は，抗CD3抗体＋抗CD28抗体刺激にしても増加しないが，抗CD3抗体＋抗CD28抗体＋抗ICOS抗体刺激により有意に増加した．これらの実験結果から，DBA/J1-ICOS$^{+/+}$F6ではICOS-B7RP-1系がIL-17産生誘導を介して関節炎の病態形成に寄与していることが示唆される[12]．

これら2つのCIAモデルから，ICOS-B7RP-1系阻害は炎症性サイトカイン産生あるいは抗タイプIIコラーゲン抗体産生抑制により関節炎に対する治療効果を発揮すると考えられる．

3）気管支喘息モデル

C57BL/6Jマウスに卵白アルブミン（ovalubumin：OVA）を腹腔内投与し（感作期，day0），day8に2% OVAの噴霧を5分間吸入，day15からday21まで1% OVAを20分間吸入（誘発期）させる気管支喘息モデルが知られている．このモデルでは，day15の吸入3時間後にマクロファージの浸潤が，day21の吸入3時間後に好酸球の浸潤が最も強く誘発される．ICOSおよびB7RP-1の発現はday0においては少量であるが，ICOS発現はday15の吸入後にピークを示し，day21まで持続する．一方B7RP-1発現はday8，15，21と漸増する．抗ICOS中和抗体を投与すると（day0，8，15〜21のOVA投与30分前に腹腔内投与），day21のOVA吸入後の気管支洗浄液（Broncho-Alveolar Lavage Fluid：BALF）中のリンパ球および好酸球数はそれぞれ50%，70%低下した．ICOS-IgまたはCTLA-4-Ig

を投与した場合も，同様なリンパ球および好酸球数の低下がみられた．さらにこのモデルではメサコリン吸入により気道抵抗が上昇するが，抗ICOS中和抗体，ICOS-IgまたはCTLA-4-Igの投与により気道抵抗の上昇はほとんど消失した．抗ICOS抗体を感作期（day0，8のみ）に投与した場合と，誘発期（day21）のみに投与した場合を比較したところ，BALF中のリンパ球・好酸球数，気道抵抗上昇は誘発期のみの中和抗体投与で改善した．これとは対照的に，CTLA-4-Igの場合は感作期の投与では改善したが，誘発期の投与では効果を認めなかった．抗ICOS抗体（day21のみ）またはCTLA-4-Igの投与（day0，8のみ）により，day21のOVA吸入後のBALF中のIL-4・IL-5・IL-10・IL-13の低下を認めた．Day21のOVA吸入前後でリンパ節細胞を分離しケモカインレセプターおよびサイトカインのmRNAレベルでの発現を定量PCR法で測定したところ，OVA吸入によりCCR3，CCR4，CCR8，IL-4，IL-10の発現は増強された．OVA吸入前の抗ICOS抗体投与により，これらの発現はほぼ完全に抑制され，IFN-γの発現が増強された．以上の結果から，この気管支喘息モデルでのT細胞感作期にはCD28-B7系が，誘発期にはICOS-B7RP-1系が重要なはたらきをしていると考えられる．また，ICOS-B7RP-1系はTh2反応を促進するとともに，所属リンパ節でのCCR3，CCR4，CCR8発現を増強する．これらのケモカインレセプターのリガンドであるエオタキシン（eotaxin），MDC，I-309などがアレルギー性炎症反応において肺へのTh2細胞の浸潤に関与することが知られていることから，ICOS-B7RP-1系はケモカインレセプターの発現を介して肺へのTh2細胞のリクルートを制御するはたらきもあると考えられる[13]．

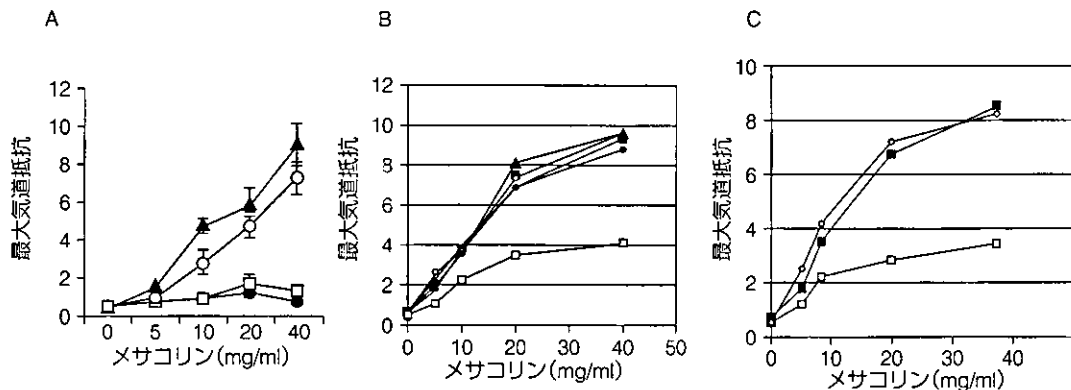気管支喘息モデルでは，ICOSが調節性T細胞（T$_R$細胞）の分化に関与することも報告されている．BALB/cマウスに3日間連続でOVAを経鼻投与（吸入）後，4日目に気管支リンパ節から分離

図 3. 気管支喘息モデルにおける ICOS-B 7 RP- 1 系の作用（Akbari, O. *et al*, 2002[14]より改変引用）

A. BALB/c マウスに OVA を腹腔内投与(OVA による感作)した 8 日後に OVA を 3 日間連続で経鼻投与すると，メサコリン吸入による気道抵抗の増強(OVA による気道過敏性の誘発)が認められる．しかし，*in vitro* で 3 回刺激した OVA 特異的 T_R 細胞を OVA 腹腔内投与の 8 日後に移入し，その 48 時間後から OVA を 3 日間連続で経鼻投与すると気道抵抗の増強は完全に抑制される．OVA 特異的 T_R 細胞移入前に抗 IL-10 抗体を投与すると，T_R 細胞による抑制効果は消失する．●無処置，○OVA による感作，□OVA による感作＋T_R 細胞移入，■OVA による感作＋T_R 細胞移入＋抗 IL-10 抗体投与．

B. OVA により感作・気道過敏性誘発をおこなうと気道抵抗の増大が認められる(○)が，OVA 経鼻投与によりトレランスを誘導したマウスでは，OVA による感作・気道過敏性誘発後の気道抵抗の増大は軽減する(□)．しかし，OVA 経鼻投与時に抗 ICOS 抗体(▲)または抗 B 7 RP-1 抗体(■)を投与すると，トレランスは誘導されず，OVA による感作・気道過敏性誘発後に気道抵抗は増大する．●はトレランス誘導なしで抗 B 7 RP-1 抗体を投与した場合．

C. OVA 経鼻投与 1 回後の気管支リンパ節樹状細胞を移入されたマウス(□)では，トレランスが誘導され，OVA による感作・気道過敏性誘発後の気道抵抗の増大は軽減する．しかし，移入時に樹上細胞を抗 B 7 RP-1 抗体で処理する(■)とトレランスは誘導されず，OVA による感作・気道過敏性誘発後に気道抵抗は増大する．PBS 経鼻投与後の気管支リンパ節樹状細胞を移入されたマウス(○)ではトレランスは誘導されず，OVA による感作・気道過敏性誘発後の気道抵抗は増大する．

した樹状細胞を用いて，OVA 特異的 T 細胞レセプターを有する DO 11.10 細胞を刺激すると IL-10 と IL-4 の両者が発現される．しかし，同じ刺激を毎週 1 回，合計 4 回くり返すと，DO 11.10 細胞は次第に IL-4 を発現しなくなり IL-10 の発現は逆に亢進してくる．これらの IL-10 発現 OVA 特異的 T 細胞は OVA による気道過敏性誘導を阻害する T_R 細胞として作用する．BALB/c マウスに OVA を腹腔内投与(OVA による感作)した 8 日後に OVA を 3 日間連続で経鼻投与すると，メサコリン吸入(気道過敏性の誘発)による気道抵抗の増強が認められる．しかし，上記の方法で 3 回刺激した OVA 特異的 T 細胞を OVA 腹腔内投与の 8 日後に移入し，その 48 時間後から OVA を

3 日間連続で経鼻投与すると気道抵抗の増強は完全に抑制される(図 3 A)．病理組織学的にも炎症細胞浸潤や気管支上皮細胞増生などの所見が，T_R 細胞の移入によりほぼ完全に消失する．この T_R 細胞の作用は，T_R 細胞と同時に抗 IL-10 抗体を投与することにより消失する(図 3 A)．上記の実験系において T_R 細胞を移入されたマウスでは 3 日間の OVA 経鼻投与後の recipient の CD 4[+]・IL-4 産生 T 細胞が減少し，CD 4[+]・IL-10 産生 T 細胞が増加する．また，OVA 特異的 T 細胞を *in vitro* で樹状細胞と OVA にて刺激すると活発に分裂・増殖を示すが，T_R 細胞を加えることにより，OVA 特異的 T 細胞の分裂・増殖は強く抑制される．この抑制は抗 IL-10 抗体または抗 B 7 RP-1 抗体の

同時添加により解除されることから，T_R細胞による OVA 特異的 T 細胞に対する増殖抑制作用は IL-10 および ICOS-B 7 RP-1 系依存的であることが示唆される．

一方，BALB/c マウスに OVA を 3 日間(day 0, 1, 2)経鼻投与するとトレランスが誘導される．トレランス誘導をおこなわずに OVA により感作・気道過敏性誘発をおこなうと気道抵抗の増大が認められるが，トレランスを誘導したマウスでは，OVA による感作・気道過敏性誘発後の気道抵抗の増大は軽減する．しかし，OVA 経鼻投与時に抗 ICOS 抗体または抗 B 7 RP-1 抗体を投与するとトレランスは誘導されず，OVA による感作・気道過敏性誘発後の気道抵抗は増大する．すなわち，ICOS-B 7 RP-1 系の阻害によりトレランスの誘導がおこなわれなくなる(図 3 B)．*In vitro* 解析のため蛍光ラベルした OVA 特異的 T 細胞を移入後に OVA 経鼻投与によりトレランスを誘導したマウスでは，移入された OVA 特異的 T 細胞の IL-4・IL-10 の発現増強が認められるが，移入時に抗 B 7 RP-1 抗体を同時投与すると，IL-4 の発現はほぼ不変で，IL-10 の発現が約 1/30 に抑制される．同時に OVA 特異的 T 細胞の分裂回数も増加する．この実験から OVA 経鼻投与によるトレランスの誘導には ICOS-B 7 RP-1 系による IL-10 の発現誘導および OVA 特異的 T 細胞の分裂抑制が重要であることが示される．

さらに OVA 経鼻投与 1 回後の気管支リンパ節樹状細胞には B 7 RP-1 が発現し，また解析のために蛍光ラベルした OVA 特異的 T 細胞移入後に OVA 経鼻投与を 3 回おこなうと，移入された OVA 特異的 T 細胞は 2 週間のあいだ ICOS を強く発現する．OVA 経鼻投与 1 回後の気管支リンパ節樹状細胞を移入されたマウスでは，OVA による感作・気道過敏性誘発後の気道抵抗の増大は軽減する．しかし，移入時に樹上細胞を抗 B 7 RP-1 抗体で処理するとトレランスは誘導されなくなる(図 3 C)．

これらの実験結果から，①トレランス誘導性樹状細胞は B 7-1・B 7-2 とともに B 7 RP-1 を発現し，抗原特異的 T 細胞を刺激することにより IL-10 を選択的に発現する T_R 細胞を誘導する，②この T_R 細胞が他の抗原特異的 T 細胞の増殖・IL-4 産生を抑制し IL-10 産生を誘導する，③その結果，気道免疫応答が抑制されるというシナリオが描かれる[14]．

おわりに

以上，3 つの病態モデルにおける ICOS-B 7 RP-1 系の解析結果を解説した．これらの論文から，ICOS-B 7 RP-1 系は免疫応答成立後(efferent phase)には，促進的因子として作用するが，免疫応答誘導期(antigen priming phase)には抑制的因子として作用する場合があることが示された．抑制的因子として作用する場合には，EAE モデルで示された Th 1 反応への偏移に加え，気管支喘息モデルで示されたトレランス誘導も重要な作用機序と考えられる．Efferent phase における ICOS-B 7 RP-1 系の作用がヒトの免疫性疾患においても確認されれば治療の新たな標的となりうる．一方，トレランス誘導における ICOS-B 7 RP-1 系の作用を利用することにより，抗原特異的なヒトの免疫性疾患治療法の開発が進展する可能性が考えられる．今後，ヒトの免疫性疾患における ICOS-B 7 RP-1 系の関与が順次報告されてくると予想され[15)16)]，ICOS-B 7 RP-1 系の制御がそれらの疾患における新規治療法開発と結びつくことが期待される．

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＋TAKEUCHI Tsutomu/埼玉医科大学総合医療センター第二内科

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Regulation of *Chk2* gene expression in lymphoid malignancies: involvement of epigenetic mechanisms in Hodgkin's lymphoma cell lines

N Kato[1], H Fujimoto[1], A Yoda[1], I Oishi[1], N Matsumura[1], T Kondo[2], J Tsukada[3], Y Tanaka[3], M Imamura[4] and Y Minami[*,1]

[1] Department of Genome Sciences, Faculty of Medical Sciences, Graduate School of Medicine, Kobe University, 7-5-1, Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan
[2] Department of Gastroenterology and Hematology, Hokkaido University, Graduate School of Medicine, Kita 15 Nishi 7, Kita-ku, Sapporo, Hokkaido 060-8638, Japan
[3] First Department of Internal Medicine, University of Occupational and Environmental Health, School of Medicine, Kitakyushu 807-8555, Japan
[4] Department of Hematology and Oncology, Hokkaido University, Graduate School of Medicine, Kita 15 Nishi 7, Kita-ku, Sapporo, Hokkaido 060-8638, Japan
* Corresponding author: Y Minami, Department of Genome Sciences, Faculty of Medical Sciences, Graduate School of Medicine, Kobe University, 7-5-1, Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan. Tel: +81-78-382-5560; Fax: +81-78-382-5579; E-mail: minami@kobe-u.ac.jp

Abstract

The tumor suppressor Chk2 kinase plays crucial roles in regulating cell-cycle checkpoints and apoptosis following DNA damage. We investigated the expression levels of the genes encoding Chk2 and several cell-cycle regulators in nine cell lines from lymphoid malignancies, including three Hodgkin's lymphoma (HL) lines. We found that all HL cell lines exhibited a drastic reduction in Chk2 expression without any apparent mutation of the *Chk2* gene. However, expression of Chk2 in HL cells was restored following treatment with the histone deacetylase inhibitors trichostatin A (TsA) and sodium butyrate (SB), or with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5Aza-dC). Chromatin-immunoprecipitation (Chip) assays revealed that treatment of HL cells with TsA, SB or 5Aza-dC resulted in increased levels of acetylated histones H3 and H4, and decreased levels of dimethylated H3 lysine 9 at the *Chk2* promoter. These results indicate that expression of the *Chk2* gene is downregulated in HL cells via epigenetic mechanisms.

cyclin-dependent protein kinase inhibitor; FCS, fetal calf serum; HDAC, histone deacetylase; HL, Hodgkin's lymphoma; H/RS, Hodgkin and Reed-Sternberg; IR, ionizing radiation; LFS, Li-Fraumeni syndrome; MSP, methylation-specific PCR; PMSF, phenylmethyl sulfonyl fluoride; SB, sodium butyrate; TsA, trichostatin A

Introduction

Chk2, the mammalian homolog of the yeast *Rad53* and *Cds1* genes, encodes a nuclear serine/threonine kinase that plays a crucial role in the DNA damage response and helps guard the integrity of the genome by regulating cell-cycle checkpoints, DNA repair and apoptosis.[1–3] Following DNA damage, Chk2 is phosphorylated and activated by ataxia-telangiectasia-mutated (ATM)-dependent and -independent mechanisms,[4–8] and exerts diverse biological effects via phosphorylation of its downstream effector molecules, including p53, Cdc25, BRCA1, PML and E2F-1.[9–14] Importantly, germ-line mutations within the *Chk2* gene have been reported in a subset of families with Li-Fraumeni syndrome (LFS), but who have a normal, wild-type *p53*,[15–17] suggesting that Chk2 may function as a tumor suppressor. While mutations in the *Chk2* gene have been implicated in occasional sporadic tumors,[3,18–22] somatic *Chk2* mutations are rare in particular in lymphoid malignancies.[23–25]

Lymphoid malignancies are a heterogeneous group of neoplasms characterized by distinct clinical, immunophenotypical, morphological, cytological and genetic features. Alterations in tumor suppressors that are involved in the DNA damage response have been reported to be frequently involved in the pathogenesis of lymphoid malignancies. In particular, the incidence of *p53* mutations is apparently high in lymphoid malignancies, and is associated with the aggressive clinical development of these neoplasms.[26,27] It has been reported that AT patients have a 100-fold increased risk for the development of lymphoid malignancies.[28] Furthermore, mutations in the *ATM* gene have been associated with the development of chromosomal instability in a subset of lymphoid malignancies as a result of impairment in the DNA damage response[29,30] Since Chk2 acts downstream of ATM and upstream of p53, it is anticipated that mutations in Chk2 may also be involved in the onset or pathogenesis of lymphoid neoplasms.

In those infrequent cases where somatic *Chk2* mutations do occur in sporadic tumors, including lymphoid malignancies, it has been found that nonsense or missense mutations often result in downregulated Chk2 expression or impaired kinase activity.[20,31] It has also been recently reported that alterations in the post-transcriptional regulation of *Chk2* expression occur

in a subset of aggressive non-Hodgkin's lymphomas (HLs).[32] In these cases, Chk2 protein is poorly expressed or absent, yet *Chk2* mRNA is apparently expressed normally compared to other types of lymphomas, and no apparent mutation or altered methylation is found in the *Chk2* gene. On the other hand, in HL, malignant mononuclear Hodgkin and multinucleated Reed-Sternberg (H/RS) cells are occasionally found to harbor genetic and/or epigenetic alterations (e.g. mutation, splicing variants and DNA methylation) in genes encoding several cell-cycle regulators, including p16^{INK4a}, p14^{ARF} and IκBα.[33–36] While p53 mutations are rather rare in H/RS cells,[37] the expression of genes or proteins involved in the DNA damage response in these cells has not been thoroughly examined.

Accumulating evidence demonstrates the importance of post-translational modification of histone proteins (i.e. acetylation, methylation and phosphorylation), in addition to DNA methylation, as epigenetic mechanisms involved in the organization of chromosomal domains and gene regulation.[38–41] It is now generally appreciated that hyperacetylated histones H3 and H4 and methylated H3-lysine 4 are associated with activated genomic regions at both the local and global levels, while hypoacetylation of histones H3, H4 and methylation on H3-lysine 9 results in gene repression and silencing. Interestingly, it has been shown that there exists an interplay between DNA methylation and histone modification (methylation and acetylation). In fact, methylation of histone H3-lysine 9, associated with aberrant gene silencing of the *p14ARF/p16INK4a* locus in tumor cells, is rapidly reversed by treatment with a DNA methyltransferase inhibitor, 5-Aza-2'-deoxycytidine (5Az-dC).[42]

In this study we first examined the expression levels of *Chk2*, *p53* and *Wip1* in nine cell lines from various lymphoid malignancies, including three HL lines. Chk2 mRNA and protein expression was dramatically lower in all three HL cell lines compared to the other cell lines, yet there was no apparent mutation in the *Chk2* gene. Therefore, we tested the possibility that epigenetic mechanisms are involved in this aberrant expression of the *Chk2* gene in these cells using the histone deacetylase (HDAC) inhibitors trichostatin A (TsA) and sodium butyrate (SB). Histone acetylation was assayed by a chromatin-immunoprecipitation (Chip) method. Our results indicate that decreased expression of the *Chk2* gene in HL cells is due, at least in part, to hypoacetylation of histones H3 and H4. We further investigated the possible hypermethylation of the *Chk2* gene in HL cells by DNA methylation assays, but found that the global methylation patterns in the *Chk2* gene and the methylation status of a likely methylation site, a CpG-rich region upstream of the *Chk2* gene, were unaffected in HL cells. However, treatment of HL cells with 5Az-dC resulted in the hypomethylation of histone H3-lysine 9 and hyperacetylation of histones H3 and H4, leading the restoration of normal *Chk2* expression. These findings indicate that DNA methylation of *Chk2* at an as-yet unidentified site(s) is also involved in the downregulation of *Chk2* expression, and that there exists an interplay between DNA methylation and histone modification in regulating the expression of *Chk2* in HL cells. We also discuss the possible relationship between downregulation of *Chk2* expression and the oncogenic properties of HL cells.

Results

Expression of *Chk2* mRNA and protein in cell lines from HLs and other lymphoid malignancies

We first performed Northern blot analysis to examine the expression levels of *Chk2*, *p53* and *Wip1* mRNAs in nine cell lines from various lymphoid malignancies; HDLM2 and L428 (HLs, nodular sclerosis), KM-H2 (HL, mixed cellularity), KM3 (pre-B lymphocytic leukemia), RL (non-HL), FL318 and FL518 (follicular lymphomas), Black93 (Burkitt's lymphoma), and Jurkat (T cell leukemia) (see Materials and Methods). Although the expression patterns of the *Chk2*, *p53* and *Wip1* mRNAs were different among the various cell lines examined, each transcript was detected at the predicted size, with the exception of *p53* mRNA in HDLM2 and Jurkat cells (Figure 1a). Interestingly, all the three HL cells, HDLM2, L428 and KM-H2 cells, exhibited drastically decreased expression of *Chk2* mRNA compared to the other six cell lines (Figure 1a). The *Chk2* genes from HL cells (L428 and KM-H2 cells) and from KM3 and RL cells were not found to possess any apparent mutations within their exons by sequence analyses (data not shown). These results suggest that the *Chk2* mRNA is altered at the transcriptional or post-transcriptional level in HL cells.

Decreased expression of *Chk2* mRNA in HL cells would presumably result in the decreased expression of Chk2 protein. To verify this possibility, Chk2 protein expression was analyzed by anti-Chk2 immunoblots of whole-cell lysates. As shown in Figure 1b, Chk2 protein expression levels correlate generally with *Chk2* mRNA levels in the cell lines examined, and decreased expression of Chk2 protein was observed in all three HL cells compared to other cell lines. It is possible that downregulation of Chk2 expression may be
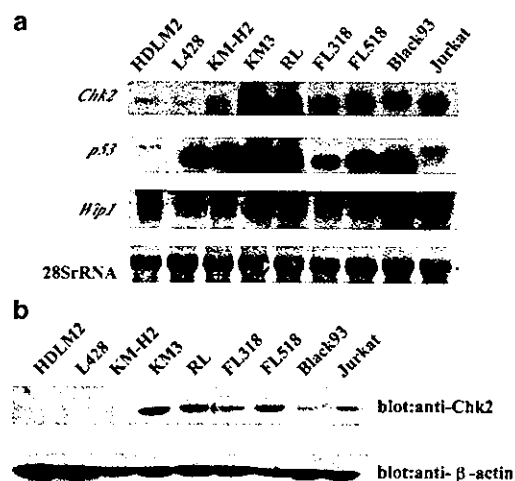

Figure 1 Expression levels of Chk2 mRNA and protein in cell lines from various malignancies. (a) Expression levels of *Chk2*, *p53* and *Wip1* mRNAs in the indicated cell lines derived from various lymphoid malignancies were determined by Northern blot analysis as described in Materials and Methods. Filters were stained with methylene blue to visualize 18S and 28S ribosomal RNAs (*28SrRNA* was indicated at the bottom panel). (b) Expression of Chk2 and β-actin proteins in the indicated cell lines was determined by anti-Chk2 or anti-β-actin immunoblotting of whole-cell lysates (equal protein amounts) from the respective cell lines as described in Materials and Methods

involved in the resistance to apoptosis and the occurrence of aneuploidy frequently observed in HLs (see Discussion).[36]

Effect of HDAC inhibitors (TsA and SB) on Chk2 expression in HL cells

In order to clarify the mechanisms of altered *Chk2* gene expression in HL cells, we tested the effect of the HDAC inhibitors TsA and SB on *Chk2* expression in HL cells. HL cell lines (L428, KM-H2 and HDLM2 cells) and several other cell lines (KM3 and RL cells) were treated with either TsA or SB for 2–3 days or left untreated, and the levels of *Chk2*, *p53* and *Wip1* mRNA expression were determined by Northern blot analysis. Intriguingly, treatment of HL cells with SB resulted in increased expression of *Chk2* mRNA, but to levels lower than those observed in untreated KM3 and RL cells (Figure 1a and 2a, data not shown). Treatment of L428 and KM-H2, but not HDLM2 cells, with TsA also enhanced *Chk2* expression to lesser extents compared to that with SB, indicating differential sensitivities of the respective HL cells toward TsA and SB. The results are consistent with the idea that hypoacetylation of histones is responsible for the downregulation of *Chk2* expression in HL cells. On the other hand, treatment of HL cells with TsA or SB caused a decrease in *p53* and *Wip1* mRNA (Figure 2a), indicating that some form of epigenetic regulation is ultimately responsible for *p53* and *Wip1* expression in HL cells. These results suggest that aberrant gene expression of *Chk2*, *p53* and *Wip1* in HL cell lines is due, at least in part, to epigenetic regulation, that is, acetylation of histones.

We next examined expression of the Chk2 proteins in the three HL cell lines following treatment with TsA or SB (Figure 2b). Treatment of L428 and KM-H2 cells with TsA or SB resulted in augmented expression of Chk2, as determined by immunoblot analysis (Figure 2b). Treatment of HDLM2 cells with SB, but not TsA, also caused an increase in Chk2 expression. Thus, in HL cell lines treated with these inhibitors, protein expression correlates with expression of each mRNA. This suggests that expression of these proteins is regulated, at least in part, by epigenetic mechanisms at the transcriptional level.

Effect of DNA methyltransferase inhibitor 5Aza-dC on Chk2 expression in HL cells

We next examined the effect of the DNA methyltransferase inhibitor 5Aza-dC on Chk2 expression (mRNA and protein) in HL cells (Figure 3a). To this end, HL cell lines (L428, KM-H2 and HDLM2 cells) were treated with 5Aza-dC for 2–3 days or left untreated, and the levels of *Chk2* mRNA and Chk2 protein were determined by Northern blot and immunoblot analyses, respectively. As shown in Figure 3a (upper panel), treatment of HL cells with 5Aza-dC resulted in increased expression of *Chk2* mRNA, but to levels lower than those observed in untreated KM3 and RL cells (data not shown), suggesting that DNA methylation is also involved in the downregulation of *Chk2* expression in HL cells. In agreement with *Chk2* mRNA expression, treatment of HL cells with 5Aza-dC also resulted in augmented expression of Chk2 protein, suggesting that
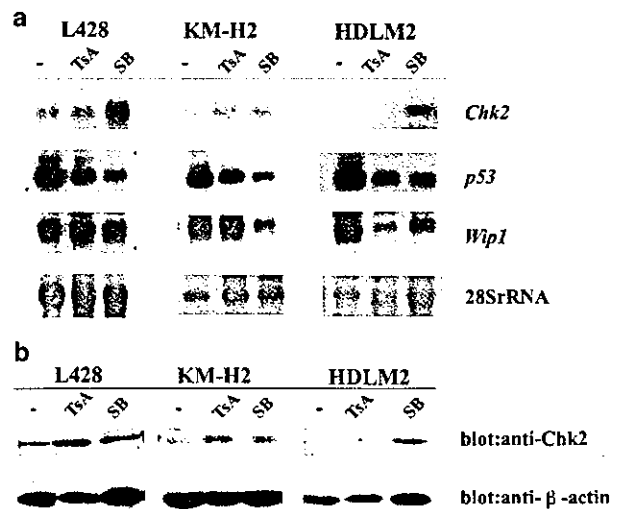

Figure 2 Expression of Chk2 mRNA and protein in HL cells (L428, KM-H2 and HDLM2) cells after treatment with TsA or SB. (a) HL cells (L428, KM-H2 and HDLM2 cells) were treated or not treated with TsA or SB for 2–3 days as described in Materials and Methods. Expression of *Chk2*, *p53* and *Wip1* mRNAs was determined by Northern blot analysis as described in Materials and Methods. Filters were stained with methylene blue to visualize 18S and 28S ribosomal RNAs (*28SrRNA* was indicated at the bottom panel). (b) HL cells (L428, KM-H2 and HDLM2 cells) were treated or not treated with TsA or SB for 2–3 days as described in Materials and Methods. Expression of Chk2 and β-actin proteins in the respective cells under the indicated conditions was determined by anti-Chk2 or anti-β-actin immunoblotting of whole-cell lysates (equal protein amounts) as described in Materials and Methods

expression of Chk2 protein is regulated, at least in part, by epigenetic mechanisms at the transcription level.

Methylation status of *Chk2* gene in HL and other malignant lymphoid cell lines

The effects of 5Aza-dC on *Chk2* gene expression in HL cell lines suggest that methylation of cytosine residues at CpG dinucleotides in the *Chk2* gene may be involved in the regulation of *Chk2* gene expression in these cells. To test this possibility, we examined overall methylation patterns in the *Chk2* gene in HL (L428 and KM-H2 cells) and other cell lines (KM3 and RL cells). Genomic DNA from each cell line was digested with an isoschizomeric restriction enzyme pair; *Hpa*II, which is methylcytosine-sensitive, and *Msp*I, which is methylcytosine-resistant, or with *Hind*III as a control, and Southern blot analysis was performed as described in Materials and Methods. As expected, the genomic DNA from all the four cell lines digested with *Msp*I or *Hind*III exhibited identical band patterns, while that digested with *Hpa*II exhibited a different pattern depending on the cell line (Figure 3b). It was also found that the overall methylation status of the *Chk2* gene in HL and other cell lines does not correlate with the expression levels of *Chk2* mRNA.

Database analysis revealed the presence of a CpG-rich region upstream of a putative transcription initiation site of *Chk2* gene (Figure 3c). Therefore, we examined the methylation status within this CpG-rich region in L428 and KM3 cells using a methylation-specific PCR (MSP) method employing
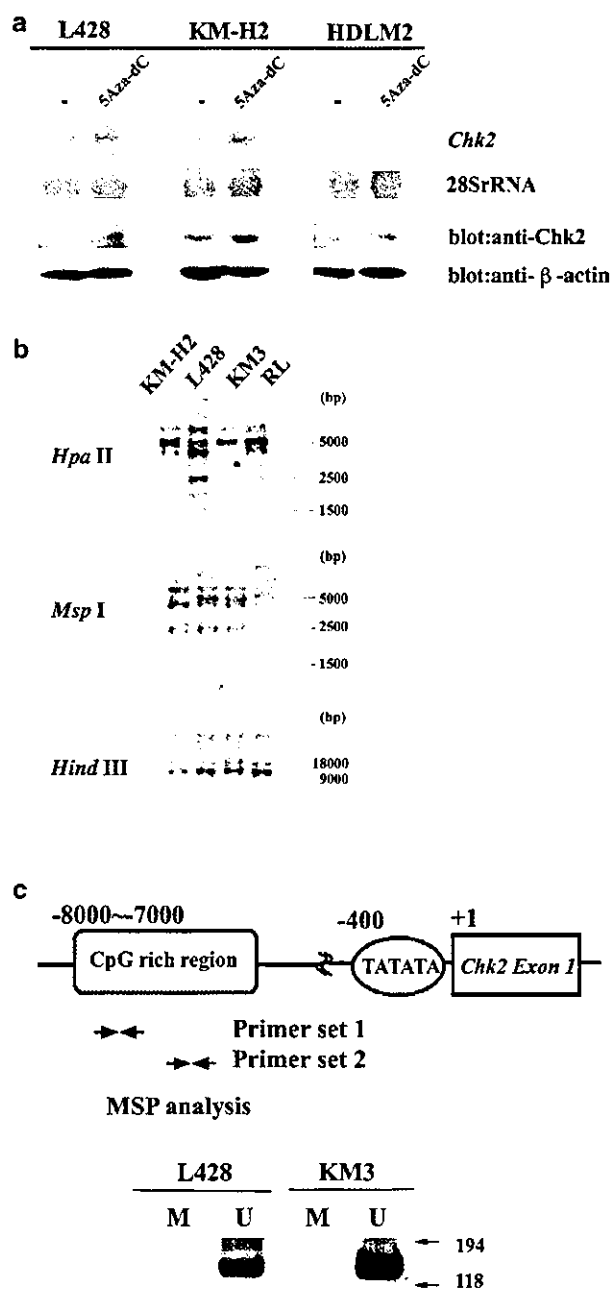
a   L428   KM-H2   HDLM2

Chk2

28SrRNA

blot:anti-Chk2

blot:anti-β-actin

b

(bp)

Hpa II

- 5000
- 2500
- 1500

(bp)

Msp I

- 5000
- 2500
- 1500

(bp)

Hind III

18000
9000

c

-8000~7000   -400   +1

CpG rich region   TATATA *Chk2 Exon 1*

Primer set 1
Primer set 2

MSP analysis

L428   KM3

M   U   M   U

194
118

Figure 3 Methylation status of the *Chk2* gene in HL cells. (a) HL cells (L428, KM-H2 and HDLM2 cells) were treated or not treated with 5Aza-dC for 2–3 days as described in Materials and Methods. Expression of *Chk2* mRNA was determined by Northern blot analysis, and expression of Chk2 and β-actin proteins was determined by anti-Chk2 or anti-β-actin immunoblotting of whole-cell lysates (equal protein amounts) as described in Materials and Methods. (b) Overall methylation pattern of the *Chk2* gene was examined by Southern blot analysis using full-length cDNA probe for *Chk2* as described in Materials and Methods. The molecular marker is shown to the right. (c) Methylation status within the CpG-rich region of *Chk2* gene was determined by MSP as described in Materials and Methods. Locations of the CpG-rich region and the MSP primer sets (primer sets 1 and 2) are indicated (upper panel). Bisulfite-treated DNAs from L428 and KM3 cells were amplified using two unmethylated (U) and methylated (M) DNA-specific primer sets. Amplification with the primer set 2 (U and M) is indicated (lower panel)
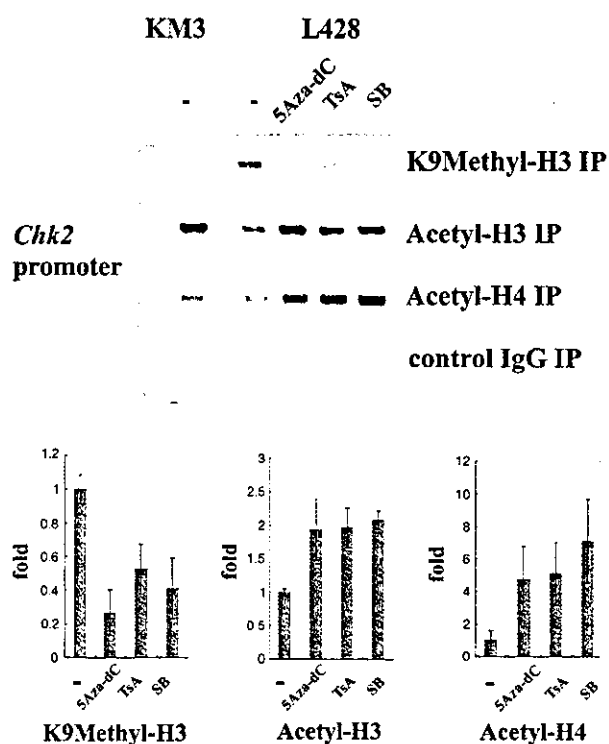
KM3   L428

K9Methyl-H3 IP

Chk2   Acetyl-H3 IP
promoter
Acetyl-H4 IP

control IgG IP

1.2   3   12
1   2.5   10
0.8   2   8
0.6   1.5   6
0.4   1   4
0.2   0.5   2
0   0   0

K9Methyl-H3   Acetyl-H3   Acetyl-H4

Figure 4 Levels of acetylated histone H3 and H4 and dimethylated histone H3 lysine 9 at the *Chk2* promoter in L428 and KM3 cells after treatment with 5Aza-dC, TsA or SB. L428 and KM3 cells were treated with 5Az-dC, TsA, or SB or left untreated for 2–3 days as described in Materials and Methods. Chip was performed with antidimethyl histone H3 (K9), antiacetyl histone H3, antiacetyl histone H4 or control antibodies, and the immunoprecipitated DNA was subjected to PCR with primers specific to the promoter region of the *Chk2* gene as described in Materials and Methods. Chip PCR products were separated in 2% agarose gels, and stained with ethidium bromide. Histograms show quantitation of band intensity of the respective Chip PCR products (lower panel). Data are expressed as the mean ± S.D. (relative to the levels in untreated cells (−, mock treated)) in four independent experiments

two MSP primer sets (primer sets 1 and 2) (Figure 3c). Genomic DNA from L428 and KM3 cells produced a strong PCR product with unmethylated sequence-specific primers, but not with the methylated sequence-specific primers (primer set 1). MSP analysis of the genomic DNA from both L428 and KM3 cells using primer sets 2 also amplified only the unmethylated sequence (data not shown). Thus, the CpG-rich region of *Chk2* in both L428 and KM3 cells appears to be unmethylated.

Effect of 5Aza-dC TsA and SB on histone acetylation and methylation at the *Chk2* promoter in L428 and KM3 cells

To examine the acetylation status of the histones H3 and H4 associated with *Chk2* gene, in particular the *Chk2* promoter, in HL cells, we performed Chip using polyclonal antibodies against acetylated histone H3 and acetylated histone H4. We examined untreated KM3 cells, untreated L428 cells, and TsA- or SB-treated L428 cells as described in Materials and Methods. As shown in Figure 4, we observed that histone H3

(and histone H4) associated with the *Chk2* promoter was hypoacetylated in untreated L428 cells compared to untreated KM3 cells. Treatment of L428 cells with either TsA or SB resulted in hyperacetylation of both histones H3 and H4, suggesting that decreased expression of the *Chk2* gene in the HL cell line L428 is due, at least in part, to hypoacetylation of histones H3 and H4.

Since it has been shown that methylation of histone H3-lysine (K) 9 is associated with inactive genes, we also examined the methylation status of this histone by Chip assay. Methylation of *Chk2* promoter-associated H3-K9 was clearly higher in untreated L428 cells compared to untreated KM3 cells (Figure 4). This suggests that hypermethylation of histone H3-K9 may also be involved in repression of *Chk2* gene expression in L428 cells. Accumulating evidence demonstrates an interplay between DNA methylation and histone modification (methylation and acetylation).[42] The methyl-CpG binding protein, MeCP2, associates with HDAC complexes, suggesting a mechanism by which histone modification can be induced by changes in DNA methylation.[43,44] Interestingly, treatment of L428 cells with 5Aza-dC resulted in the drastic inhibition of histone H3-K9 methylation and enhancement of histones H3 and H4 acetylation, showing that there is indeed an interplay between DNA methylation and histone modification. Collectively, these results indicate the crucial role of epigenetic mechanisms in downregulating *Chk2* gene expression in the HL cell line, L428 cells.

Apoptotic changes induced by γ-irradiation in L428 cells by treatment with SB

Recent studies have shown that *Chk2*^{-/-} mice and cells have decreased susceptibility to ionizing radiation (IR).[45,46] It has also been reported that HL cells frequently exhibit resistance to apoptosis.[36] Therefore, it is possible that the observed alteration in the expression of *Chk2* and related genes in HL cells may be involved in apoptosis resistance. To examine this possibility, we examined the effect of SB on the susceptibility of HL cells (L428 and KM-H2 cells) to apoptosis induced by γ-irradiation, as described in Materials and Methods. L428 cells were treated with SB, or left untreated, subjected to γ-irradiation (15 Gy), and labeled 24 h later with PI and annexin V. PI/annexin V dual staining indicated that the percentages of annexin V[high] PI^{high} cells relative to total cells in untreated L428 cells increased from 4 to 14% in response to γ-irradiation, while the percentages in SB-treated L428 cells increased from 5 to 27% (Figure 5). Similar results were obtained when KM-H2 cells were treated with SB (data not shown). These results imply that upregulation of *Chk2* and other related genes in HL cells by SB treatment contributes to the increased susceptibility of HL cells to γ-irradiation.

Discussion

Chk2 kinase plays a central role in signaling in response to DNA damage, functioning downstream of ATM and upstream of p53.[2,3] Following DNA damage, Chk2 is phosphorylated and activated by ATM-dependent and -independent mechanisms,[4–8] and this activated Chk2 then phosphorylates and
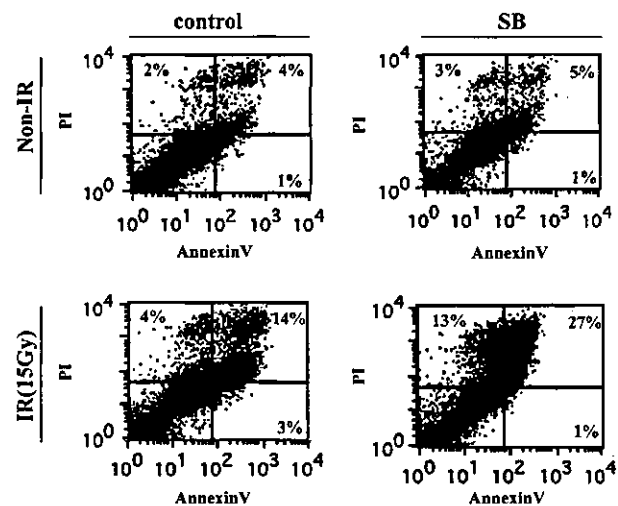

Figure 5 Detection of apoptotic changes induced by γ-irradiation in L428 cells treated with SB. L428 cells were treated with SB (final conc. 1 mM) or left untreated for 2–3 days, subjected to γ-irradiation (15 Gy), and subsequently cultured for 24 h. Cells were labeled with FITC-conjugated annexin V mAB and PI, and analyzed by flowcytometry as described in Materials and Methods. Numbers indicate percentages of annexin V[high] PI[high], annexin V[high] PI[low] and annexin V[low] PI^{high} cells relative to total cells. Essentially identical results were obtained in two independent experiments

stabilizes p53.[1–3,10,11] This, in turn, leads to induction of the *p21* and *Wip1* genes, which encode a cyclin-dependent protein kinase inhibitor (CKI) p21, and the oncogenic nuclear protein phosphatase Wip1, respectively.[47–49] Interestingly, Wip1 has been shown to mediate negative feedback regulation of p38–p53 signaling in response to DNA damage.[50] In addition, it has been well documented that most if not all molecules involved in DNA damage response signaling are tumor suppressors, and mutations of these genes have been implicated in a wide variety of tumors. In fact, germ-line mutations of the *Chk2* gene have been identified in a subset of families affected by LFS,[15–17] although somatic *Chk2* mutations are rather rare, particularly in lymphoid malignancies.[23–25]

In our present study, we found that cell lines from HLs exhibit drastically decreased expression of the *Chk2*, but not *p53* and *Wip1*, compared to cell lines from other lymphoid malignancies. We found that this decreased expression was due, at least in part, to altered epigenetic regulation (Figures 1 and 2). In particular, downregulation of *Chk2* gene expression was shown to involve hypoacetylation of histones H3 and H4, hypermethylation of histone H3-lysine (K) 9 and DNA hypomethylation at an as-yet-unidentified site(s) outside the CpG-rich region in the *Chk2* promoter (Figures 3 and 4). Although a recent study has shown that aberrant post-transcriptional regulation of *Chk2* expression may occur in a subset of aggressive non-HLs,[32] we believe our data represent the first example of altered epigenetic regulation of *Chk2* gene expression in cells from malignant tumors.

HLs are characterized by the presence of malignant mononuclear H/RS cells, surrounded by reactive cells attracted by cytokines and chemokines, which are abundantly

produced by H/RS cells.[51] It has been reported that constitutive activation of nuclear factor (NF)-κB and AP-1 are also implicated in HLs,[52–54] and that genetic and/or epigenetic alterations (e.g. mutation, splicing variant and DNA methylation) in genes encoding cell-cycle regulators, including p16[INK4a], p14[ARF] and IκBα, exist in H/RS cells from HLs.[33–36] Furthermore, a recent study using tissue-microarrays has revealed that Cyclin E, Cdk2, 6, Stat3, Hdm2, Bcl2, Bcl-XL, Survivin, molecules involved in cell-cycle regulation and prevention of apoptosis, are overexpressed in HLs, explaining their high proliferative activity and resistance to apoptosis.[36] Intriguingly, polymorphic mutations of the *ATM* gene have recently been identified in childhood Hodgkin's disease.[55] These mutated *ATM* genes encode functionally abnormal ATM proteins, suggesting the involvement of polymorphic variations of the *ATM* gene in the pathogenesis of childhood HD, presumably due to a defect in cell-cycle checkpoint regulation. Our findings, that altered epigenetic regulation of *Chk2* gene in HL cells results in the downregulation of Chk2 kinase and abrogates DNA damage response signaling in the cells (Figure 5, see below), suggest that this novel molecular feature of HLs that may also be useful for the diagnosis of the disease. In fact, downregulated expression of Chk2 and abrogation of DNA damage response could also account for the resistance to apoptosis and aneuploidy occasionally observed in HLs.

Our results demonstrate that downregulation of *Chk2* gene expression in HL cell lines is due partly, if not entirely, to altered epigenetic regulation of the *Chk2* gene, that is, hypoacetylation of histones H3 and H4, hypermethylation of H3-K9 and hypomethylation of DNA (Figures 2, 3a and 4). Although overall DNA methylation analysis of the *Chk2* gene and MSP analysis of the CpG-rich region of *Chk2* failed to detect a DNA methylation site(s) within the *Chk2* gene that differs between HL and cells from other lymphoid malignancies (Figures 3b and c), our results with 5Aza-dC (see Figures 3a and 4) nevertheless indicate the involvement of DNA methylation in decreased *Chk2* expression in HL cells. Further study will be required to identify potential crucial cytosine residues within the *Chk2* gene. Our results with 5Aza-dC also provide an example of the interplay between DNA methylation and histone modification (methylation and acetylation)[42] (Figure 4). This interplay can be assumed to be mediated by a molecular mechanism whereby the methyl-CpG binding protein, MeCP2, associates with HDAC complexes.[43,44] However, a recent study using a genetic approach has indicated that histone modification can occur independently of DNA methylation, and that DNA methylation serves to 'lock in', rather than initiate gene repression.[56] Further study will be required to clarify this issue. Moreover, it has been appreciated that epigenetic regulation of gene expression can be affected by environment surrounding cells. Thus, the epigenetic regulation in particular cells *in vitro* and *in vivo* may be different. Since our present study is restricted to established HL cell lines, it will be of importance to examine the epigenetic regulation of the *Chk2* gene by utilizing fresh specimens from patients with HLs.

It has been shown that targeted disruption of the *Chk2* gene results in decreased susceptibility of cells in response to IR, and that HL cells occasionally exhibit resistance to

apoptosis.[36,45,46] Consistent with these observations, we found that upregulation of *Chk2* in HL cells (L428 and KM-H2 cells) following treatment by the HDAC inhibitor SB resulted in increased susceptibility of the cells to IR (Figure 5). At present we do not know to what extent SB-induced *Chk2* expression contributes to this increased susceptibility. Future studies examining the effects of ectopically expressed Chk2 on the susceptibility to IR in HL cells might address this question. Importantly, our results may also provide some insight into the potential therapeutic use of HDAC inhibitors against malignant tumors, especially on the combined treatments (e.g. HDAC inhibitor and IR) for patients with HLs.

Materials and Methods

Cell lines and culture conditions

Three HL cell lines and six other lymphoid malignancies cell lines were analyzed. The HL cell lines were L428, HDLM2 (both nodular sclerosis),[57] and KM-H2 (mixed cellularity),[57] and the other cell lines were KM3 (pre-B lymphocytic leukemia),[58] RL (non-HL),[59] FL318 and FL518 (follicular lymphomas),[60] Black 93 (Burkitt's lymphoma),[61] and Jurkat (T cell leukemia).[62] All cell lines were maintained in RPMI1640 supplemented with 10% (v/v) fetal calf serum (FCS). L428 cells were treated with 5Aza-dC (5Aza-dC, Sigma) (final conc. 5 μM), TsA (Sigma) (final conc. 300 nM), or SB (Aldrich) (final conc. 1 mM) for 2–3 days.

Northern blot analysis

Cells were harvested and total RNA was prepared using the ISOGEN RNA preparation kit (Wako). Total RNA (10 μg) was electrophoresed in 1% agarose formaldehyde gels and transferred onto nylon membranes (Nytran N, Schleicher & Schuell) as described previously.[63] Probes were labeled with [α-^{32}P]dCTP (Amersham, 3000 Ci/mmol) using the Multiprimer labeling kit (Amersham) and were hybridized using PerfectHyb (TOYOBO) following the manufacturer's instructions. Specific activity was approximately 2 × 10^6 cpm/ng for each of the probe DNAs. 28S rRNA was visualized by staining filters with methylene blue. The probe DNAs were prepared as follows: *Chk2*,[1] 1.7 kbp *EcoRI–XhoI* fragment from pcDNA3-*Chk2*; *p53*,[64] 1.8 kbp *BamHI* fragment from pCMV-*p53*; *Wip1*,[50] 1.8 kbp *BglII–SphI* fragment from pCMV-*Wip1*.

Southern blot analysis

Genomic DNAs were prepared from the four cell lines (L428, KM-H2, RL and KM3) by phenol–chloroform extraction. To detect methylation sites within genomic DNA, genomic DNA (10 μg) from each cell line was digested with *HpaII* (New England Biolabs) or *MspI* (TAKARA). Both restriction enzymes recognize the same sequence 'CCGG', but *HpaII* cannot digest the methylated sequence, while *MspI* can.[65,66] *HpaII*- or *MspI*-digested DNAs were electrophoresed in 0.8% agarose gels, and transferred onto nitrocellulose membranes (PROTRAN BA 85 CELLU-LOSENITRAT, Schleicher & Schuell). Probes were labeled as described in Northern blot analysis. After hybridization, the membranes were washed with 5 × SSC/0.1% SDS at 65°C.

Methylation-specific PCR analysis

To perform MSP,[67] genomic DNA (2 μg) was treated with sodium bisulfite using the DNA Modification Kit (CpG Genome, Intergen), following the

manufacturer's instructions. Bisulfite converts unmethylated cytosines, but not methylated cytosines, to uracil. The sequences of the MSP primers were as follows. Primer set 1: unmethylated DNA (U); 5′-GTTTTTTTTTTTTGTAGGTTAGATTTTGAT-3′ and 5′-CTTCAACCTTA TAAACTAATACAAACAACA-3′. methylated DNA (M); 5′-GTTTTT TTTTTTCGTAGGTTAGATTTCGAC-3′ and 5′-CTTCAACCTTATAAAC TAATACGAACGACG-3′. Primer set 2: (U); 5′-TTGTTTGTGATGTAG TATTGTAGTTTAGTG-3′ and 5′-ACAAATAACCACAACTAAATAAAC CACCAA-3′. (M); 5′-TCGTTTGCGACGTAGTATCGTAGTTAGCG-3′ and 5′-ACGAATAACCACGACTAAATAAACCGCCGA-3′. The sequences recognized by these primers were located within a likely methylation site, a CpG-rich region upstream of the putative transcription initiation site of the Chk2 gene. Following PCR, the respective samples were fractionated on 2% agarose gels and visualized under UV.

Protein preparation and immunoblotting

Cells were harvested and solubilized with lysis buffer (50 mm Tris-HCl (pH 7.4), 0.5% (v/v) NP-40, 150 mm NaCl, 5 mm EDTA, 50 mm NaF, 1 mm Na$_3$VO$_4$, 1 mm phenylmethyl sufhonyl fluoride (PMSF), 10 μg/ml leupeptin and 10 μg/ml aprotinin), and cell lysates were prepared by centrifugation at 12 000 $\times$ g for 15 min at 4°C. Amounts of proteins in the respective cell lysates were quantitated by the Bio-Rad DC protein assay kit (Bio-Rad). Cell lysates containing equal amounts of proteins were separated by SDS-PAGE (10% PAG), and transferred onto PVDF membrane filters (Immobilon, Millipore). The membranes were immunoblotted with rabbit polyclonal anti-human Chk2 (HF, NK, TK, YM, unpublished data), anti-p53 (DO1, Santa Cruz), or mouse monoclonal anti-β-actin (AC-15, Sigma-Aldrich) antibodies, and bound antibodies were visualized with HRP-conjugated goat anti-rabbit IgG or goat anti-mouse IgG antibodies (Bio-Rad) using a chemiluminescence reagent (Western Lightning, Perkin Elmer Life Sciences) following the manufacturer's instructions. Rabbit polyclonal anti-Chk2 antibody was raised against peptides corresponding to amino acids 523–543 of human Chk2.

Chip assay

In total, 1 $\times$ 10^6 cells were treated with 1% formaldehyde for 15 min at 37°C to crosslink proteins to DNA. Subsequently, chromatin was solubilized and subjected to sonication to obtain DNA fragments with an average size of 750–1000 bp. Chip assays were performed as described previously[68] with an equal amount (5 μl) of antiacetyl histone H3, antiacetyl histone H4, antidimethyl histone H3 (K9) antibodies (Upstate Biotechnology) or normal rabbit IgG (Capel) as a control. The immunoprecipitated DNA was analyzed by PCR with primers specific to the Chk2 promoter region. The sequences of the primers utilized were 5′-GACGGAGTTTCACTATGTTGGCC-3′ and 5′-CTGCCATGAGACTGCT GAGCCTCAACAT-3′, respectively.

Analysis of apoptosis

Cells treated with or without SB were subjected to γ-irradiation (15 Gy) and cultured for 24 h. Cells were harvested and labeled with annexin V and PI using the TACS Annexin V-Apoptosis Detection kit (R&D systems) following the manufacturer's instructions and subsequently analyzed by a flow cytometry (FACSCalibur, Becton Dickinson). The percentages of cells in each fraction were analyzed using the software CellQuest.

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