Molecular mechanisms of aflatoxin B₁ hepatocarcinogenesis

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Aflatoxin B₁ (AFB₁) is produced by *Aspergillus flavus* related fungi that grow on improperly stored foods such as corn, rice and peanuts¹⁾. It is widely known that AFB₁ is one of the environmental risk factors for human liver cancer especially in South-east Asia, China and sub-Saharan Africa²⁻⁴⁾. AFB₁ requires metabolic conversion to its epoxide in order to damage DNA^{5,6)}. As shown in Fig.1, the carcinogenic AFB₁-8,9-epoxide reacts with guanine to form a number of adducts, and one reasonable model is that these adducts or secondary DNA lesions derived from them, lead to heritable genetic changes that induce malignant transformation. Despite the structurally various population of DNA adducts that forms in cells treated with the toxin, the mutational spectrum of the toxin is dominated by one genetic change of the transversion form GC to TA⁶⁾. Similarly, the high frequency of G to T mutation of the tumor suppressor gene p53 at the third base position of codon 249 was reported in human hepatocellular carcinomas from these endemic areas^{7,8)}. Although the mechanisms of AFB₁ activation and metabolism in liver cells have been reported by many groups including our laboratory⁹⁻¹⁴⁾, the detailed mechanism of AFB₁ hepatocarcinogenesis is still unknown. To understand the molecular mechanism of AFB₁ hepatocarcinogenesis, we have established the hepatoma cell lines designated as Kagura-1 (K1) and Kagura-2 (K2) cells from AFB₁-induced rat hepatoma¹³⁾. Here we report that the 14-3-3 β gene plays a pivotal role in abnormal growth of K2 cells *in vitro* and *in vivo*.

Introduction to AFB₁ hepatocarcinogenesis in rat

The cancer arises through multistep processes associated with progressive accumulation of genetic damage by the action of chemical carcinogens within a single cell line leads to a progressively cellular appearance, deregulated cell growth, and, finally carcinogenesis. Loss of function mutation of tumor suppressor genes such as *Rb*, *p53* and deregulated activation and mutation of oncogenes such as *ras* family and *c-myc*, were implicated in tumorigenicity and metastasis. In rat hepatomas induced by AFB₁, overexpression of the oncogenic *c-myc* gene⁹⁾ and point mutation of *ras* family genes^{10,11)} have been reported. In the course of the study of AFB₁ hepatocarcinogenesis, we found that the expression of the 14-3-3 β gene was deregulated together with the *c-myc* gene in K1 and K2 cells^{12,13)}.

Fig. 1. Metabolic activation of AFB₁ leads to DNA adduct formation

AFB₁ is metabolized by cytochrome P450 to the AFB₁-8,9-epoxide, which form the DNA adduct. AFB₁-guanidine-DNA adduct, destabilizes glycosidic bond and depurinates to the apurinic (AP) site shown. Alternatively, the primary adduct can suffer opening of its imidazole ring, giving rise to the chemically and biologically stable formamidopyrimidine (FAPY) adduct, AFB₁-FAPY.

Mutation of 14-3-3 β gene locus was also detected in these cells¹⁴. On the other hand, the mutation and/or deregulated expression of *ras* family oncogenes and suppressive oncogene such as *p53* and *Rb* were not detected in K1 and K2 cells¹⁴. Thus, it is highly possible that 14-3-3 β gene cooperates with *c-myc* gene in cellular transformation.

Effect of enforced expression of antisense 14-3-3 β RNA on cell growth

The 14-3-3 proteins are highly conserved and found in broad range of organisms¹⁵. These proteins have attracted interest because they participate in important cellular events including cell division and apoptosis¹⁶. The 14-3-3 proteins are phosphoserine-specific binding protein and regulate the various signal transduction pathways including malignant transformation¹⁷. To elucidate the implication of 14-3-3 β in tumor cell growth, we established K2 cell sublines in which endogenous expression of 14-3-3 β RNA was suppressed by the transfection with antisense 14-3-3 β cDNA expression vector¹⁸. The growth rates of these transfectants in medium supplemented with 1 % FCS were diminished to less than half as compared with that of the parental K2 cells. The anchorage-independent growth in the soft agar was also reduced in these transfectants. These results suggest that 14-3-3 β protein participates in the growth control of K2 cells.

Cooperation among 14-3-3 β , *raf-1* and *c-myc* in K2 cell growth

It has been reported that the maintenance of active conformation of Raf-1 kinase by the association with 14-3-3 protein is required for Ras/MAPK signal transduction pathway¹⁹. Moreover, the expression of *c-myc* gene, which is deregulated in K2 cells, synergizes with *ras*, *raf-1* and *bcl-2* oncogenes in cell transformation^{20, 21}. We examined, therefore, the effect of antisense 14-3-3 β , *raf-1* and *c-myc* oligodeoxynucleotides (ODNs) on the K2 cell growth to clarify the mechanism of abnormal cell

growth. The proliferation of K2 cells was efficiently inhibited by the antisense 14-3-3 β , *raf-1* and *c-myc* ODNs dependent on the amount of ODNs added, and their inhibition levels were similar to each other. To examine the cooperation among 14-3-3 β , *raf-1* and *c-myc* in K2 cell growth, K2 cells were treated with the various combinations of the corresponding antisense ODNs. The combinatorial treatment with the antisense ODNs of 14-3-3 β and *raf-1* inhibited the K2 cell growth in an additive manner. While, synergistic inhibition was observed by the treatment of antisense *c-myc* ODN with antisense 14-3-3 β or *raf-1* ODNs. Simultaneous treatment with the three ODNs severely inhibited K2 cell growth in a synergistic manner. These results support the assumption that 14-3-3 β maintains the active form of Raf-1 kinase and synergizes the c-Myc function in cell proliferation.

Effect of antisense 14-3-3 β RNA expression on tumorigenicity of K2 cells

We next investigated the effect of 14-3-3 β on the tumorigenicity by the K2 cells. Parental K2 cells can develop solid tumors when transplanted into flanks of nude mice. To assess the effect of suppression of 14-3-3 β expression on the tumorigenicity of K2 cells, antisense 14-3-3 β cDNA expression vector-introduced transfectants were injected into nude mice. These antisense transfectants formed solid tumors much smaller than those of parental K2 cells.

To elucidate the suppression mechanism of tumorigenicity observed in the antisense transfectants, we histochemically examined tumors induced by K2 and antisense transfectants. Since suppression of apoptosis and induction of angiogenesis are essential for tumor growth and metastasis^{22,23}, we further examined the extent of apoptosis and angiogenesis of tumors derived from the parental K2 cells and antisense transfectants. The results showed that the apoptotic cells were increased in tumors derived from antisense transfectants as compared with those from parental K2 cells. On the other hand, microvessel density was significantly decreased in tumors derived from antisense transfectants. Without the ability to recruit new blood vessels, it is likely that most tumors could not grow beyond a limited size²⁴. Thus, it seems that tumors continuously produce angiogenic factors, permitting tumor cell growth and expansion. It has been reported that angiogenic factors such as VEGF and IGF-II implicate in angiogenesis during hepatocarcinogenesis²⁵. We analyzed the expression level of VEGF mRNA in the antisense transfectants by Northern blotting. In the transfectants, the expression levels of VEGF mRNA were markedly down-regulated as compared with K2 cells. While, the expression of IGF-II mRNA was hardly detected in any K2 cells. Therefore, it is highly possible that the deregulated expression of 14-3-3 β gene largely participates in tumorigenic angiogenesis through the constitutive stimulation of VEGF mRNA expression, and the severe inhibition of tumorigenicity together with apoptosis observed in tumors formed by the antisense transfectants is caused, at least in part, by the inhibition of angiogenesis. However, at present we cannot rule out the possibility that anti-apoptotic function of 14-3-3 β implicates in tumorigenicity through the suppression of apoptotic factors such as BAD, forkhead family transcription factor FKHRL and ASK-1 kinase.

Over-expression of 14-3-3 β mRNA in various tumors

These data described above strongly supported the idea that 14-3-3 β gene could be classified as a

proto-oncogene. We examined, therefore, the expression level of 14-3-3 β mRNA in 21 kinds of tumor cell lines including hepatoma, kidney tumors, peripheral nerve tumors, glioblastoma and neuroblastoma cells. The high levels of 14-3-3 β mRNA expression were detected in all tumors examined as compared with those of corresponding adult rat tissue except for the mammary tissue. These results suggest that deregulated expression of 14-3-3 β gene participates in malignant transformation of various types of tumors as well as AFB₁-induced rat hepatoma K2 cells. Therefore, the 14-3-3 β gene could be classified as a proto-oncogene and its deregulated expression may implicate in malignant transformation of various types of tumors.

Conclusion

We found that the reduction of over-expressed 14-3-3 β protein by the enforced expression of antisense 14-3-3 β RNA caused the severe suppression of K2 cell proliferation and tumorigenicity *in vitro* and *in vivo*. It is quite possible that 14-3-3 β synergizes with c-Myc in cell transformation through the activation of Raf-1 kinase. Thus, the activation of *c-myc* and 14-3-3 β genes might be critical steps in AFB₁ hepatocarcinogenesis. The deregulated expression of 14-3-3 β gene largely participates in tumorigenicity through the induction of angiogenesis and inhibition of apoptosis.

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アフラトキシン B₁ によるラット肝発癌の分子メカニズム

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アフラトキシン B₁ (AFB₁) は、土壌菌である *Aspergillus flavus* 等によって産生されるカビ毒で、強い肝毒性と共に発癌性を有しており、実験動物に肝癌を誘発することが知られている。しかし、その詳細な発癌メカニズムに関しては不明な点が多い。そこで当研究室では、AFB₁ 誘発ラット肝癌より樹立した Kagura-2 (K2) 細胞を用いて肝発癌の分子メカニズムについて解析を行っている。K2 細胞における癌関連遺伝子について解析したところ、癌遺伝子である *c-myc* 遺伝子およびリン酸化セリン結合タンパク質である 14-3-3 β 遺伝子の発現が亢進していることが明らかとなった。今回、K2 細胞の増殖および造腫瘍性における 14-3-3 β の機能を解析した。14-3-3 β mRNA に対するアンチセンスオリゴヌクレオチドで K2 細胞を処理したところ、その増殖能は低下した。14-3-3 β は Bad に結合してアポトーシスを制御すると共に、*c-myc* と同様に細胞増殖を制御する Raf-1 キナーゼの活性を調節していることが報告されている。そこで、K2 細胞に 14-3-3 β 、*c-myc*、*raf-1* のアンチセンスオリゴヌクレオチドを様々な組み合わせで添加し、増殖能に対する作用を調べたところ、*c-myc*、14-3-3 β および *raf-1* に対するアンチセンスオリゴヌクレオチドの同時処理により、K2 細胞の増殖は顕著に抑制された。さらに、アンチセンス 14-3-3 β cDNA 発現ベクターを導入することにより 14-3-3 β の発現量を低下させた K2 細胞株を樹立し、ヌードマウス皮下へ移植したところ、腫瘍形成能の著しい低下が見られた。形成された腫瘍の性質を調べたところ、アポトーシスの増加と血管新生能の低下が認められた。以上のことから、AFB₁ により誘導される肝発癌において 14-3-3 β は *c-myc* と協同して Raf-1 キナーゼの活性を正に制御して肝癌細胞の増殖を促進するとともに、アポトーシスの抑制と VEGF 遺伝子の発現亢進による血管新生の促進を介して、造腫瘍性に関与していることが強く示唆された。

キーワード: アフラトキシン B₁, 肝癌, *c-myc*, 14-3-3 β , VEGF