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약학석사학위논문

CD133, CD44, ALDH1의 하이콘텐츠
모니터링에 기초한 아플라톡신 B1 유발
간암 줄기세포 정량

Crosstalk-eliminated Quantitative Determination of
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Based on Concurrent Monitoring of CD133, CD44, and
Aldehyde Dehydrogenase1

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이 논문을 약학석사학위논문으로 제출함

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Abstract

Crosstalk–eliminated quantitative determination of
aflatoxin B1–induced hepatocellular cancer stem
cells based on concurrent monitoring of CD133,
CD44, and aldehyde dehydrogenase1

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Cancer stem cells (CSCs), known as tumor initiating cells, have become a critically important issue for cancer therapy. The properties of CSCs, such as anticancer drug and radiation resistance, have been considered to be the source of postoperative recurrence and metastasis. In this work, it was found that hepatocellular CSCs were produced from HepG2 cells by aflatoxin B1–induced mutation, and their amount was quantitatively determined using crosstalk–eliminated multicolor cellular imaging based on quantum dot (Qdot) nanoprobe and an acousto–optical tunable filter (AOTF). Although much research has demonstrated the induction of hepatocellular cancer by aflatoxin B1 found in contaminated foods and the

interrelation between them, the formation of hepatocellular CSCs caused by aflatoxin B1 and their quantitative determination are hardly reported. Hepatocellular CSCs were acquired from HepG2 cells via magnetic bead-based sorting and observed using concurrent detection of three different markers: CD133, CD44, and aldehyde dehydrogenase1 (ALDH1). The DNA mutation of HepG2 cells caused by aflatoxin B1 was quantitatively observed via absorbance spectra of aflatoxin B1-8,9-epoxide-DNA adducts. The percentages of hepatocellular CSCs formed in the entire HepG2 cells were determined to be $9.77 \pm 0.65\%$, $10.9 \pm 1.39\%$, $11.4 \pm 1.32\%$, and $12.8 \pm 0.7\%$, respectively, at 0 μM , 5 μM , 10 μM , and 20 μM aflatoxin B1. The results matched well with those obtained utilizing flow cytometry, which is used as a general tool for the detection of hepatocellular CSCs. This study demonstrates that accurate quantitative measurement of aflatoxin B1-induced hepatocellular CSCs can be accomplished using a constructed multicolor cellular imaging system that eliminates crosstalk among hepatocellular CSC biomarkers.

Keyword

Hepatocellular cancer stem cell, Aflatoxin B1, High-content cellular imaging, Quantum dot, Diagnosis markers

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List of Abbreviations

HCC: hepatocellular carcinoma

CSCs: cancer stem cells

Qdot: quantum dot

AOTF: Acusto optic tunable filter

ALDH1: aldehydedehydrogenase 1

PBS: Phosphate Buffer Saline

DTT: dithiothreitol

1. Introduction

Mycotoxins are fungal secondary metabolites that possess various toxic effects in humans and animals. Among the mycotoxins discovered, some mycotoxins, such as ochratoxin A and sterigmatocystin, have been known to be carcinogenic [1], particularly aflatoxins have received great attention due to their high toxicity and carcinogenicity [2]. Filamentous fungi such as *Aspergillus flavus* and *A. parasiticus* are the major pathogens of food crops, which secrete aflatoxin B1 as the secondary metabolite. Consumption of aflatoxin contaminated agricultural products like rice, corn, nuts, milk and feed affects the health of both animals and humans [3]. Furthermore, it has been reported that airborne exposure to mycotoxin contaminated dust and fungal components causes several magnitude toxic effect than oral exposure in experimental mice [4, 5]. Aflatoxin B1 has been shown to be the most potent natural carcinogen, as the main aflatoxin produced by toxigenic strains [6]. The primary target organ of aflatoxin B1 is the liver. Hence, the chronic dietary exposure constitutes a significant risk factor that causes hepatocellular carcinoma (HCC). Aflatoxin B1 has been reported to be metabolized to the highly reactive intermediate aflatoxin B1-8,9-epoxide by hepatic cytochrome P450 enzymes. This intermediate binds to DNAs and intracellular

macromolecules. The unstable aflatoxin B1-8,9-epoxide is intercalated between DNA base pairs and forms N7 guanine adducts [7, 8]. Predominant mutation caused by exposure to aflatoxin B1 has been found to exhibit G to T transversion through numerous in vitro and in vivo models. The formed aflatoxin B1-8,9-epoxide-DNA adducts lead to the mutation of proto-oncogene and tumor suppressor genes, and induce HCC when the mutation is not repaired properly by DNA repair enzymes [9].

Early diagnosis of HCC constitutes an essential issue to prolong the lifetime of liver cancer patients. DNA sequencing has been utilized mainly to analyze the mutagenic effects of aflatoxin B1 on genomic DNAs. It has been verified via DNA sequence analysis of HCC patient samples that exposure to aflatoxin B1 causes a G to T mutation at the third position of codon 249 in the p53 tumor suppressor gene [10]. The identification of HCC markers with high sensitivity and specificity is another effective way to diagnose HCC [11]. α -fetoprotein, proteantigens, such as heat shock protein and glypican-3, enzymes and isozymes, and cytokines are being utilized as tumor markers for HCC [12, 13]. Particularly, detection of α -fetoprotein, as well as iconography and pathology observation, is used generally for clinical diagnosis of liver cancer. α -fetoprotein has been shown to be well-expressed in HCC. Flow cytometry and western blot

assays have been employed for the diagnosis of HCC. However, α -fetoprotein marker does not provide satisfactory results in the early diagnosis of HCC, especially α -fetoprotein-negative HCC. In spite of advances in the early diagnosis and treatment of HCC, the prognosis of patients having advanced HCC is still unfavorable [14]. Recently, tumor-initiating cells, termed as cancer stem cells (CSCs), have received great attention in cancer therapy due to their anticancer drug and radiation resistance. CSC theory assumes that only a small subset of cells possesses the ability to initiate and maintain tumor growth [15]. The resistive properties of CSCs have been considered to be the source of postoperative recurrence and metastasis [16]. Functional liver CSCs have been reported to be found in HCC cell lines. In addition, CD133 has been used as a CSC surface marker for the isolation and detection of liver CSCs [17].

Cancer diagnosis utilizing quantum dot (Qdot) nanoprobe-based high-content cellular imaging offers various advantages which may be appropriate for clinical application of early cancer diagnosis. Concurrent detection of cancer markers at the single cell level can provide promising information regarding the identification, pathological condition, and prognosis of cancer [18–20]. Qdots possess excellent physical properties, such as high sensitivity and strong photostability as imaging nanoprobe [21]. In addition, Qdot

nanoprobes are much more suitable than conventional dyes for the simultaneous detection of cancer markers because of their narrow wavelength range of emission spectrum and larger absorbance at shorter wavelengths in the range of ultraviolet to visible wavelengths [22]. These properties enable concurrent monitoring of many Qdots through excitation at a single wavelength and enable more accurate cancer diagnosis.

In this work, for the first time, quantitative high-content determination of aflatoxin B1-caused hepatocellular CSCs is reported. The quantitative determination was executed using a hypermulticolor cellular imaging system based on an acousto-optical tunable filter (AOTF) and Qdot nanoprobes [23]. AOTF-based hypermulticolor cellular imaging was utilized very efficiently for the identification of hepatocellular CSCs induced by aflatoxin B1. This was achieved by spectral overlap-free simultaneous monitoring of CD133, CD44, and aldehyde dehydrogenase 1 (ALDH1) markers, which are specific to hepatocellular CSCs.

2. Materials and Methods

A. Cell culture

HepG2 cells were purchased from the Korean Cell Line Bank. HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; 11995-073, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS; 16000-044, Gibco), 5 mg/ml streptomycin, 5 mg/ml penicillin, and 10 mg/ml neomycin (PSN; 15640-055, Gibco) under 5% CO₂ at 37°C.

B. Aflatoxin B1 treatment

Aflatoxin B1 was purchased from Sigma-Aldrich. Before the aflatoxin-B1 treatment, cells were cultured overnight so that they were in a log phase. Then, the cell culture medium was changed to serum and antibiotic-free DMEM for 6 h before the experiment. The aflatoxin-B1 stock solution was prepared in DMSO. Cells were treated with aflatoxin-B1 as a function of concentration (5 μ M, 10 μ M, and 20 μ M) for 48 h at 37°C under 5% CO₂. The final DMSO concentration in the cell culture medium was adjusted to under 0.1%.

C. Cell sorting using microbeads

One of the representative CSC markers is CD133. HepG2 cells were sorted to select hepatocellular CSC. The sorting buffer was composed of 47.3 mL 1xPBS, 200 μ L 0.5M EDTA, and 2.5 mL 10% BSA. Aflatoxin-B1-treated HepG2 cells were rinsed with 1xPBS and treated with the accutase enzyme for 20 min at room temperature. The treated cells were then detached from the surface of the cell culture plate. Then, the detached cells were collected and centrifuged at 230 rcf for 3 min. The supernatant was removed, and the pellet was resuspended in the sorting buffer of 80 μ L and CD133 microbead solution of 20 μ L (CD133 microbead kit human; 130-100-857, MACS Miltenyi Biotec). It was then incubated for 15 min at 4°C. The cells were resuspended in 1 mL sorting buffer and centrifuged at 300 g for 10 min. The supernatant was removed, and the pellet was resuspended in the sorting buffer of 500 μ L. The LS column (LS column; 130-042-401, MACS Miltenyi Biotec) was placed in the magnetic field of the MACS separator. The pre-separation filter (Pre-separation filter; 130-041-407, MACS Miltenyi Biotec) was put on the LS column. Then, the column and filter were rinsed with 1 mL sorting buffer three times. The resuspended cells were applied on the pre-separated filter. The column was then washed with 5 mL sorting buffer. The flushed-out fraction consisted of cells labeled with CD133

magnetically.

D. Quantum dot–antibody conjugation and imaging cytometry

CD133, CD44, and ALDH1 antibodies were conjugated to Qdot 705, Qdot 525, and Qdot 625 (Quantum Dot Conjugation Kit; Invitrogen, Carlsbad, CA, USA), respectively. The antibodies were treated with dithiothreitol (DTT) for antibody reduction to break the disulfide bond. Then, the antibodies were conjugated to maleimide–functionalized Qdot. The Qdot–antibody conjugations were incubated for 1 h at room temperature. Then, the conjugations were treated with 2–mercaptoethanol in order to remove the maleimide group. The Qdot–antibody conjugations were applied over the column for the elimination of unconjugated Qdot. The Qdot–antibody conjugation was diluted at 1:200 with 1% BSA. The sorted HepG2 cells were then treated with 4% formaldehyde for 10 min at room temperature. Then, the cells were washed with 1 mL 1xPBS and centrifuged at 230 g for 3 min. The supernatant was then discarded, and the pellet was resuspended in 1 ml 1xPBS. Then, the cells were washed again, and the pellet was treated with 0.2% saponin for 10 min at room temperature. The cells were then washed with

1xPBS twice. The washed cells were incubated with the diluted Qdot-antibody conjugations at room temperature for 2 h. After 2 h, the HepG2 cells were washed with 1 mL 1xPBS and centrifuged at 230 g for 3 min. After the centrifugation, the supernatant was discarded and washed again. The pellet was then resuspended in 1xPBS solution of 50 μ L. Then, 10 μ L cells were taken and placed in the 1.5 μ -slide vi (1.5 μ -slide vi; ibide GmbH, Am Klopferspitz 19, 82152 Martinsried, Germany). Qdot has a specific emission wavelength, so that the Qdot-antibody conjugations were detected at their own emission wavelengths. For the simultaneous detection of CD133, CD44 and ALDH1, AOTF was scanned as a function of wavelength, and the transmitted emissions of biomarkers were detected on the CCD.

E. Flow cytometry

Flow cytometric determination of aflatoxin B1-induced hepatocellular CSCs was executed as a complementary tool to compare results obtained with hypermulticolor imaging cytometry. CD44 and CD133 antibody-Qdot conjugations were employed to detect hepatocellular CSCs. HepG2 cells were treated with aflatoxin-B1 as a function of concentration (0 μ M, 5 μ M, 10 μ M, and 20 μ M) for 48 h at 37°C under 5% CO₂. The HepG2 cells were treated with accutase for 20 min

at 37°C. The attached cells were treated with 4% formaldehyde and 0.2% saponin under the same conditions used in the imaging cytometry. The cells were then treated with 1:200 diluted CD44 antibody-Qdot conjugation and CD133 antibody-Qdot conjugation for 2 h at room temperature. After 2 h, the cells were washed with 1 mL 1xPBS solution and centrifuged at 230 g for 3 min twice. The supernatant was removed, and the pellet was resuspended with 2 ml 1xPBS solution. Then, the resuspended cells were subjected to flow cytometric analysis using a FACS Calibur (FACS Calibur; BD Bioscience).

F. Determination of aflatoxin-B1 DNA adducts

HepG2 cells were treated with aflatoxin-B1 as a function of concentration (0 μ M, 5 μ M, 10 μ M, and 20 μ M) for 48 h at 37°C under 5% CO₂. Then, DNA were extracted from cells treated with aflatoxin-B1. This procedure was performed according to the manufacturer's instructions (Blood and Cell Culture DNA Mini Kit; 13323; Qiagen). First, cells were treated with accutase enzyme for detaching. Then, the detached cells were treated with a cell lysis buffer. Proteinase was added after a suitable incubation time, and lysate was loaded on the qiagen genomic tip. While cell constituents passed through the column, the extracted DNA bound to the

column. The column was then washed with a washing buffer in order to remove any remaining contaminants. DNA were eluted and precipitated with isopropanol.

Figure 1. A spectrum of quantum dots

A spectrum of a mixture solution containing quantum dots used for CD133, CD44, and aldehyde dehydrogenase1 conjugations. The emission wavelength was scanned from 500 nm to 725 nm.

3. Results

A. Crosstalk-free high-content detection of CD133, CD44, and ALDH1 Qdot-antibody conjugates

Figure 1. clearly represents how high-content detection of hepatocellular CSCs can be achieved without crosstalk among three different CD133, CD44, and ALDH1 biomarkers labelled with Qdot-antibody conjugates. The emission spectrum of a mixture solution composed of CD133, CD44, and ALDH1 Qdot-antibody conjugates was obtained as a function of wavelength from 500 nm to 748 nm with the interval of 3 nm by the constructed multicolor cellular imaging system. The CCD detector was operated coincidentally as the AOTF was scanned as a function of wavelength. The emission intensities were obtained every 1 s. Figure 1 shows the spectrum obtained from the mixture of three Qdot-antibody conjugates. Three different spectra corresponding to Qdot-antibody conjugates were clearly observed in the green, red, and near-IR emission region. The maximum emission wavelengths were found to be 525 nm, 625 nm, and 705 nm, respectively, and marked with arrows. As expected, emission wavelength ranges of Qdots were narrower than those of conventional dyes, which have more than 100 nm generally. The maximum

emission wavelengths, marked with arrows, emerged as peaks devoid of spectral overlap among Qdots. Based on this result, the wavelengths marked with arrows were selected as detection wavelengths for simultaneous monitoring of CD133, CD44, and ALDH1 biomarkers bound on the surface or nucleus of HepG2 cells. It is worth noting that the employed AOTF is capable of choosing optimized single detection wavelengths in consideration of emission intensity, as well as spectral overlap, based on its capability to transmit single wavelengths.

Figure 2. High-content detection

(a) High-content detection of hepatocellular cancer stem cells isolated through the bead sorting from HepG2 cancer cells treated with aflatoxin B1. CD133, CD44, and aldehyde dehydrogenase1 markers specific to hepatocellular cancer stem cells were concurrently detected using the antibody-quantum dot conjugates. (b) Quantitative determination of hepatocellular cancer stem cells was obtained as a function of aflatoxin B1 treatment concentration.

B. Identification of hepatocellular CSC

Figure 2. clearly shows that the number of hepatocellular CSCs increases from HepG2 cells treated with aflatoxin B1 as the treatment concentration of aflatoxin B1 increases from 0 μ M to 20 μ M. Biomarkers utilized for the detection of hepatocellular CSCs were CD133, CD44, and ALDH1. CD133 is a member of transmembrane glycoproteins that have been reported to be a marker of liver CSC. CD44 is one of the cell surface molecules involved in cell migration, adhesion, and proliferation. ALDH1 is an enzyme that oxidizes intracellular aldehyde. Hepatocellular CSCs have subtypes that include CD133-positive, CD44-positive, and ALDH1-positive. HepG2 cells treated with aflatoxin B1 were sorted using the CD133 microbeads. The collected CD133-positive cells were immune-stained with CD133 antibody-Qdot705, CD44 antibody-Qdot525, and ALDH1 antibody-Qdot625. As shown in Figure 2 (a), the sorted cells revealed that CD44 and ALDH1 were positive in addition to CD133, which accorded with the phenotype of hepatocellular CSCs. The sorted CD133-positive cells facilitated the identification of hepatocellular CSCs. Figure 2 (b) represents that the percentage of CSCs obtained from HepG2 cells quantitatively increases as a result of aflatoxin B1 treatment, compared with the 0 μ M. The percentages of CSCs were determined to be 9.7%, 10.9%, 11.4%, and 12.8%, respectively, when the

treatment concentrations of aflatoxin B1 were 0 μ M, 5 μ M, 10 μ M, and 20 μ M, respectively. The entire number of HepG2 cells was counted using a hemacytometer before the CD133 microbead sorting. The number of CD133-positive cells obtained via the sorting was counted again using an identical hemacytometer. This outcome clearly verifies that the increase in the number of hepatocellular CSCs is caused by the growing concentration of treated aflatoxin B1.

Figure 3. Absorption spectra of DNA

(a) Absorption spectra of DNAs from HepG2 cells treated with aflatoxin B1. The absorption spectra were plotted from 300 nm to 400 nm. The HepG2 cells were treated with aflatoxin B1 at different concentrations: 0, 5, 10, and 20 μ M.

(b) Quantitative determination of aflatoxin B1-8,9-epoxide-DNA adducts using Beer-Lambert law and molar absorptivity of the DNA adduct at 360 nm.

Aflatoxin B1-concentration	0μM	5μM	10μM	20μM
Absorption	0.019	0.2081	0.4507	0.7335
Aflatoxin B1-8,9-epoxide DNA adduct	1.8×10^{-6} M	1.1×10^{-5} M	2.55×10^{-5} M	3.47×10^{-5} M

Table 1. Absorbance of aflatoxin B₁-8,9 epoxide-DNA adducts

Absorbance of aflatoxin B₁-8,9 epoxide-DNA adducts formed in HepG2 cells and concentrations of DNA adducts determined at 360 nm.

C. AFB1–DNA adduct formation

Figure 3 shows the spectra of aflatoxin B1–DNA adducts that were formed in HepG2 cells as a result of treatment with aflatoxin B1 and isolation from HepG2 cells. The aflatoxin B1–DNA adducts were produced by the reaction of metabolized aflatoxin B1–8,9–epoxide with guanine of DNA in HepG2 cells. The aflatoxin B1–8,9–epoxide–DNA adduct has absorbance in the spectral range of 300–400 nm, which is transparent to pure DNAs. Maximum absorbance of the aflatoxin B1–8,9–epoxide–DNA adduct has been reported to be at 360 nm. The amount of formed aflatoxin B1–8,9–epoxide–DNA adducts was determined using the Beer–Lambert law ($A = \epsilon bc$; A :absorbance; ϵ : molar absorptivity; c :concentration; b :beam path length). The molar absorptivity of aflatoxin B1–8,9–epoxide at 360 nm is known to be 21,800 M⁻¹cm⁻¹. The genomic DNAs of HepG2 cells treated with aflatoxin B1 as a function of their concentrations were extracted and isolated. The aflatoxin B1–8,9–epoxide–DNA adducts existing in the isolated genomic DNAs were observed by a UV spectrophotometer, and their absorbance spectra were measured, as shown in Fig 3(a). The absorbances of aflatoxin B1–8,9–epoxide–DNA adducts were monitored at 360 nm to determine the amounts of formed aflatoxin B1–8,9–epoxide–DNA adducts. The absorbance values were found to be 0.019, 0.2081, 0.4507,

and 0.7335, at 0 μ M, 5 μ M, 10 μ M, and 20 μ M aflatoxin B1 treatment concentrations, respectively (Table 1). The concentrations of aflatoxin B1-8,9-epoxide-DNA adducts on the basis of the Beer-Lambert law were calculated to be 1.8×10^{-6} M, 1.1×10^{-5} M, 2.55×10^{-5} M, and 3.47×10^{-5} M, at 0 μ M, 5 μ M, 10 μ M, and 20 μ M aflatoxin B1 treatment concentrations, respectively (Table 1). Figure 3 (b) represents both the absorbance and concentration of the formed aflatoxin B1-8,9-epoxide-DNA adducts. Figure 3 (b) indicates that exposure to aflatoxin B1 induces formation of aflatoxin B1-8,9-epoxide-DNA adducts in HepG2 cells, and the degree of formation is proportional to the concentration of aflatoxin B1. It is noteworthy that the aflatoxin B1 treatment concentrations in Figure 3 are identical to those used to induce hepatocellular CSCs in Figure 1. These results indicate that aflatoxin B1-8,9-epoxide-DNA adducts were produced in large quantities in the concentration range of aflatoxin B1 treatment to such an extent that DNA adducts were not properly repaired by DNA repair enzymes.

Figure 4. Flow cytometric detection

(a)–(d) Flow cytometric detections of hepatocellular cancer stem cells acquired from HepG2 cancer cells as a function of aflatoxin B1 treatment concentration. The upper right section corresponds to the hepatocellular cancer stem cell. (e) Quantitative determination of hepatocellular cancer stem cells obtained using flow cytometry.

Group		Total cell (cell/ml)	CD133+ cell (cell/ml)	CSC (%)	Average CSC (%)	Aflatoxin B ₁ induced CSC (%)
Aflatoxin B ₁ 0μM	1	1.22×10 ⁷	1.19×10 ⁶	9.8	9.77±0.65	0
	2	4.16×10 ⁷	3.76×10 ⁷	9.1		
	3	7×10 ⁶	1.28×10 ⁵	10.4		
Aflatoxin B ₁ 5μM	1	3.8×10 ⁶	3.77×10 ⁵	9.92	10.9±1.39	1.13
	2	3.68×10 ⁷	3.79×10 ⁶	10.3		
	3	1.2×10 ⁷	1.5×10 ⁷	12.5		
Aflatoxin B ₁ 10μM	1	3.2×10 ⁶	3.33×10 ⁵	10.4	11.4±1.32	1.63
	2	4.32×10 ⁷	4.7×10 ⁶	10.9		
	3	1.92×10 ⁷	1.96×10 ⁶	12.9		
Aflatoxin B ₁ 20μM	1	2.6×10 ⁶	2.91×10 ⁵	12.1	12.8±0.7	3.03
	2	3.76×10 ⁷	4.81×10 ⁶	12.8		
	3	1.66×10 ⁷	2.24×10 ⁶	13.5		

Table 2. The percentage of hepatocellular cancer stem cell

The number of CD133 positive cells increased as a result of treatment of HepG2 cells with aflatoxin B1 and the percentage of hepatocellular cancer stem cell.

D. Flow cytometric analysis

The increase in the number of hepatocellular CSCs induced by aflatoxin B1 was observed using a flow cytometer. Flow cytometry has been utilized as a general tool for the detection of hepatocellular carcinoma. In this work, a multicolor cellular imaging system based on AOTF and Qdot nanoprobe was attempted for the first time as an advanced tool for the detection of hepatocellular CSCs caused by aflatoxin-B1. Flow cytometric detection of hepatocellular CSCs was performed to compare its result with that obtained by multicolor cellular imaging, and to verify accuracy of multicolor cellular imaging as a tool for the detection of hepatocellular CSCs. In Figure 4 (a) to (d), CD44 emission was plotted on the x-axis, while CD133 emission was plotted on the y-axis. In the x-axis, areas closer to the right side represent CD44-positive. On the other hand, areas closer to the upper side in the y-axis represent CD133-positive. Since the phenotype of hepatocellular CSCs is positive with respect to both CD44 and CD133 markers, the upper-right section corresponds to the population of hepatocellular CSCs existing in the entire HepG2 cells. The flow cytometric measurement was performed to a total of 30,000 HepG2 cells. As shown in Figure 4 (a), the hepatocellular CSC proportions were found to be 5.24%, 6.66%, 10.3%, and 11.2%, at 0 μ M, 5 μ M, 10 μ M, and 20 μ M aflatoxin B1 treatment concentrations, respectively. These

values were plotted as a bar graph in Figure 4 (e). Compared to the control, the increase of aflatoxin B1-induced CSC ratio was determined to be 1.13%, 1.63%, and 3.03%, at 5 μ M, 10 μ M, and 20 μ M, respectively (Table 2).

4. Discussion

CD133 was originally classified as a marker that identifies primitive hematopoietic and neural stem cells [24]. Since then, many reports have confirmed that CD133 are expressed in a variety of solid tumor cells. Particularly CSCs in tumors (brain, colon, prostate) have been found to contain CD133. CD133 is known to have a characteristic to decrease its expression level with tumor cell differentiation. This property causes CD133 to be a specific marker that isolates and identifies CSCs. Recently, it has been reported that CD133 silencing inhibits stemness properties and improves sensitivity to chemotherapeutic agents in CD133-positive liver CSCs [25, 26]. CD44 is a receptor for hyaluronic acid and involved in interaction between cells. CD44 also plays a role in cell migration based on interactions with ligands, such as collagens and matrix metalloproteinase. Moreover, CD44 has been reported as a cell surface marker for breast, prostate, and hepatocellular CSCs [27–29]. In addition to CD133 and CD44, ALDH1 is utilized to identify hepatocellular CSCs [30]. ALDH1 catalyzes the oxidation of aromatic aldehydes to carboxyl acids. ALDH plays an important role in oxidative stress. ALDH can remove radiation-induced free radicals and causes NAD(P)H to function as an antioxidant. Activation of ALDH is important for the regulation of radiosensitivity. In

addition, increasing the activation of ALDH1A1 and ALDH3A1 isoform makes radioresistance and chemoresistance possible [31]. Recent results reveal that CSCs are capable of being quite diverse phenotypically, as observed in breast CSCs. Both CD44+CD24[−] and CD44+CD24⁺ cell populations are monitored in breast CSCs [32]. The diverse phenotypes of CSCs require more selective observation of individual CSC markers without crosstalk for the accurate identification of CSCs. False-positive detection of a CSC marker can arise from crosstalk between CSC markers. Until now, most probing materials to detect hepatocellular CSCs are dye-labelled antibodies. These probes have been applied to flow cytometric analyses in most cases. However, most dyes have emission spectra which have a very broad wavelength range [16]. This broad emission wavelength range inherent to probing dyes considerably limits the simultaneous labels of probes to different markers for the identification of hepatocellular CSCs. In fact, the number of probes used in flow cytometric analyses has been very limited in the detection of hepatocellular CSCs. Simultaneous monitoring of more than two hepatocellular CSC markers is hardly found in flow cytometric analyses. Only CD133 monitoring has been attempted to observe hepatocellular CSCs in most cases [17]. In this work, for the first time, concurrent monitoring of CD133, CD44, and ALDH1 markers was performed

successfully for the identification of hepatocellular CSCs induced by aflatoxin B1. Specifically, this has been achieved based on Qdot probe materials and emission imaging at a single wavelength using AOTF [23]. As shown in Fig. 1, Qdots have narrow spectral ranges, which are quite advantageous for the use of a larger number of Qdots. Moreover, AOTF possesses the capability to selectively detect individual hepatocellular CSC markers without crosstalk. It is worth noting that the number of CSC markers detected concurrently can be increased to more than four with the constructed detection system. Based on these results, it can be expected that a broad range of hepatocellular CSC research can be performed as a function of its phenotype using the constructed system.

CSC possesses strong resistance to anticancer agents and enables cancer recurrence through its self-renewal capacity. Therefore, accurate CSC diagnosis is essential to achieve efficient anticancer chemotherapy without recurrence. As shown in Fig. 2, this work successfully measured a quantitative increase of hepatocellular CSCs induced by aflatoxin B1 using high-content monitoring of hepatocellular CSC markers without crosstalk among them. Aflatoxin B1 is a fungal second metabolite found in staple agricultural foods, such as rice, and well known as a carcinogen [1, 2]. Previously, numerous studies regarding aflatoxin B1-induced

liver cancer have been reported. The experimental results disclose that aflatoxin B1 is an inactive-state molecule called pro-carcinogen, and absorbed into the body via contaminated foods and then metabolized into aflatoxin B1-8,9-epoxide by cytochrome P450 enzymes in the liver. Due to the instability of aflatoxin B1-8,9-epoxide, it reacts with intracellular DNAs and generates aflatoxin B1-8,9-epoxide-DNA adducts. Predominant mutation arising from aflatoxin B1-8,9-epoxide-DNA adducts has been found to exhibit G to T transversions [7-9]. As shown in Fig. 3(a), aflatoxin B1-8,9-epoxide-DNA adducts were formed in HepG2 cells as a result of treatment with aflatoxin B1, and its amount quantitatively increased as the treatment concentration of aflatoxin B1 increased from 5 to 20 μ M. Up to now, a variety of DNA mutations have been reported, including DNA backbone breaks and cross links, as well as DNA base damage. Aflatoxin B1-8,9-epoxide-DNA adduct is one of the bulky adducts and leads to disruption of transcription or DNA replication. When DNA mutation occurs, DNA repair is executed in the nucleus. However, if DNA damage repair does not function properly, severe cellular malfunctions by disrupted or altered protein expression result in, and ultimately cause, cancer. Generally, damage tolerance can arise at serious DNA damage sites, resulting in altered expression. Translesion DNA synthesis is one of the damage

tolerances and bypasses the damage sites using replication machinery. Low-fidelity DNA polymerase is used for translesion DNA synthesis, while high fidelity DNA polymerase is employed for normal DNA replication. Low fidelity polymerase makes it possible to bypass serious DNA damage sites by adding new nucleotides randomly to the damage sites for replication. As a result of this replicating process, mutations of the newly synthesized sequence are induced. Translesion DNA synthesis and other damage-tolerance processes contribute to breaking the tight regulation of the self-renewal process [33]. Consequently, uncontrolled cell proliferation occurs. This study verified that the quantitative formation of hepatocellular CSCs arose from the mutation by aflatoxin B1. The number of hepatocellular CSCs was increased by the mutation induced by aflatoxin B1, although DNA repair enzymes exist in the nucleus of HepG2 cells. DNA mutation was quantitatively observed through the absorbance spectra of aflatoxin B1-8,9-epoxide-DNA adducts. Damage tolerance, including translesion DNA synthesis, may be one of the factors contributing to the mutation caused by aflatoxin B1-8,9-epoxide-DNA adducts. The amounts of aflatoxin B1-8,9-epoxide-DNA adducts determined by absorbance spectra were thought to be so large that the mutation led to the formation of hepatocellular CSCs. It is noteworthy that hepatocellular CSCs were quantitatively monitored in the same

concentration range in which the formation of aflatoxin B1-8,9-epoxide-DNA adducts was observed. This result reveals that the hepatocellular cancer cell line can be transformed to CSC due to the mutation caused by the treated aflatoxin B1.

5. Conclusion

This work demonstrated quantitative Qdot-based multicolor cellular imaging of hepatocellular CSCs produced by the treatment of HepG2 cells with aflatoxin B1. Intracellular simultaneous observation of many CSC biomarkers clearly provides higher diagnostic accuracy of CSC and more information regarding CSC subtype, the execution of high-content assay of even three different biomarkers has been seldom achieved up to now. In addition to selective monitoring at a particular monochromatic wavelength by AOTF, multiplex probing of Qdot successfully accomplished high-content diagnosis of CSC occurring by mutation caused by aflatoxin B1. In the identical concentration range of aflatoxin B1, the hepatocellular CSCs were formed and their number increased as a function of aflatoxin B1 concentration. The formed hepatocellular CSCs could be successfully detected based on the simultaneous monitoring of CD133, CD44 and ALDH1 without the crosstalk among them.

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국 문 초 록

아플라톡신 B1은 곡류나 우유, 씨앗 등을 보관 시 부패로 생성되는 mycotoxin 중 하나로, 인간과 동물의 발암을 유발하는 발암물질로 알려져 있다. 주로 간암을 유발하여 만성적으로 아플라톡신 B1에 노출 될 경우 간암을 유발한다는 연구 사례가 보고되었다.

종양 형성의 시작으로 알려진 암줄기세포는 항암 치료에 중요한 논제로 논의되어왔다. 또한 많은 선행 연구자들이 아플라톡신 B1의 간암 유발에 관하여 연구하였으나, 간암줄기세포의 형성과 이를 정량한 연구를 최초로 시행함에 논문의 의의가 있다.

본 연구에서는 아플라톡신 B1에 의해 변이가 유발된 HepG2 세포에서 간암 줄기세포를 검출하였고, 이러한 간암줄기세포의 증가를 정량하기 위해 Quantum dot (Qdot) 과 Acousto-optical tunable filter (AOTF)를 사용하여 crosstalk이 없는 멀티컬러 세포 이미징을 시행하였다.

간암 줄기세포는 HepG2 세포에서 마그네틱 비드를 사용해 분리하여 얻었으며, CD133, CD44, Aldehyde dehydrogenase1 (ALDH1) 세 가지의 마커를 사용하여 동시에 검출하였다. 또한 아플라톡신 B1에 의해 유발된 HepG2 세포의 DNA 돌연변이의 원인인 아플라톡신 B1-8,9-epoxide DNA adduct 형성을 흡광도 스펙트럼을 통해 정량적으로 관찰하였다.

아플라톡신 B1의 농도는 0 μ M, 5 μ M, 10 μ M, 20 μ M 로 처리하였고, 세 가지 마커로 검출한 간암줄기세포의 백분율은 9.77 ± 0.65 %, 10.9 ± 1.39 %, 11.4 ± 1.32 %, 12.8 ± 0.7 % 였다. 이 결과는 기존에 사용하던 방법인 FACS 유동세포분석기로 얻은 결

과와 일치한다.

따라서 본 연구는 아플라톡신 B1이 간암 세포를 간암 줄기세포로 변이시킴을 Qdot을 기초로 한 멀티컬러 세포 이미징 시스템을 통하여 증명하였다.

주요어

간암 줄기세포, 아플라톡신 B1, 하이콘텐트 세포 이미징, Quantum dot, 진단 마커

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