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Photosensitization of bilirubin on proliferation and DNA synthesis in ascitic hepatoma cells[1]

ZHOU Xiu-Fang, CHEN Fu-Yu, ZENG Zhao-Chong, ZHENG Rong-Liang[2]
(*Department of Biology, Lanzhou University, Lanzhou 730000, China*)

KEY WORDS bilirubin; photosensitizing agents; hepatocellular carcinoma; neoplasm DNA; free radicals

AIM: To observe the effects and its mechanism of photosensitization of bilirubin on ascitic hepatoma (Hep A) cells. **METHODS**: After the cells were illuminated under the light (1.0×10^5 lx) for 10 min, deoxy[^{3}H]thymidine was added for DNA synthesis assay. The cells were dyed with 0.5 % trypan blue and were counted. **RESULTS**: The cell mortality of illuminated groups were much higher than that of dark groups ($P < 0.01$). The inhibitions of DNA synthesis of illuminated groups were stronger than that of dark groups ($P < 0.01$). The cell mortality and inhibition of DNA synthesis of illuminated groups were positively dependent upon the concentrations of bilirubin and illuminating time. No difference of DNA synthesis between room light illumination groups and dark groups ($P < 0.05$). **CONCLUSION**: The photosensitization of bilirubin killed Hep A cells obviously. Bilirubin under room light had no effect on inhibition of DNA synthesis. The photosensitization of bilirubin was closely related to $^1O_2$ and H_2O_2 and not to OH· and O_2^-.

Bilirubin, the end product of heme catabolism in mammals, is a potentially cytotoxic, lipid soluble waste product that needs to be excreted. However, bilirubin at micromolar concentration *in vitro*, efficiently scavenges peroxyl radicals[1]. In liposomes, bilirubin suppresses the antioxidation more than α-tocopherol, which is regarded as the best antioxidant of lipid peroxidation. Thus bilirubin is a physiological, chain-breaking antioxidant[1]. Bilirubin exhibited antitumor activity[2] and acted as a photosensitizer with four pyrrole rings. Many photosensitizers have been used to treat cancer due to their photosensitization. In this paper, the photosensitization of bilirubin on DNA synthesis in ascitic hepatoma (Hep A) cells and on cell mortality were studied.

MATERIALS AND METHODS

Chemicals Bilirubin (AR, Sigma) was dissolved in 1, 2-propanediol to 1, 0.1, 0.01, 0.001 mmol·L^{-1}. Superoxide dismutase (SOD, AR, Gansu Xiahe Biological Products Factory) was prepared as a 2 g·L^{-1} aqueous solution. Catalase (Cat, AR, Sigma) was a 1 g·L^{-1} aqueous solution. Sodium azide (CR, Shanghai Chemical Agent Factory), sodium formate (AR, Beijing Chemical Factory), and mannitol (AR, Chengdu Chemical Agent Factory) were prepared in triple distilled water to 10 mmol·L^{-1}.

Illumination A water trough was placed between an iodine wolfram light (1 kW) and cell flasks for heat insulation. The trough was made of glass with a 5-mm-thick bottom. Water layer was 16 cm high.

The cells were illuminated 37 cm under this light with 1.0×10^5 lx radiant intensity. There was no illumination in control group and the flask was wrapped with black paper. The maximal absorption wave of bilirubin was 453 nm. All experiments were carried out at room temperature 20 - 25 ℃.

Measurement of DNA synthesis Hep A cells were suspended at a density of 2×10^6 cells per bottle in pH 7.3 *RPMI-1640 medium (Gibco)* containing 20 % fetal calf serum, penicillin 100 kU·L^{-1} and streptomycin 100 mg·L^{-1}. Each vial contained 5 mL medium. The cells were preincubated at 37 ℃ for 15 - 20 h and then incubated with bilirubin for 1 h. The cells were illuminated under the light for 10 min as the illumination group. The control cells were kept in dark. Deoxy[^{3}H] thymidine ([^{3}H]TdR, Shanghai Institute of Nuclear Research) was added to final concentration of 37 MBq·L^{-1}. After 2- or 24-h incubation, bilirubin was removed by centrifugation at $100 \times g$ for 10 min. The cells were washed with saline twice, and then digested with a drop of $HClO_4$ and H_2O_2 respectively at 80 ℃ for 40 min. The mixture was transferred into 10 mL of scintillant liquid and was assayed with an FJ-2100 scintillating counter.

Cell mortality The cells were dyed with 0.5 % trypan blue and counted.

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[2] Correspondence to Prof ZHENG Rong-Liang.
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Assay of hydrogen peroxide Having been illuminated for 10 min, 0.5 mL of bilirubin (38 μmol·L^{-1}) was immediately added 0.2 mL of 20 % $TiCl_4$ and 0.2 mL $NH_3 \cdot H_2O$ 17 mol·L^{-1}. A stable yellow precipitate of H_4TiO_5 indicated H_2O_2 generation[3].

RESULTS AND DISSCUSION

Photosensitization of bilirubin The cell mortalities of illuminated groups were much higher than those of dark groups ($P<0.01$). The cell mortality of illuminated groups was positively dependent upon the concentration of bilirubin. In dark groups, the mortalities were around 5 % without concentration dependence, indicating that the bilirubin *per se* had slight cytotoxicity (Fig 1).

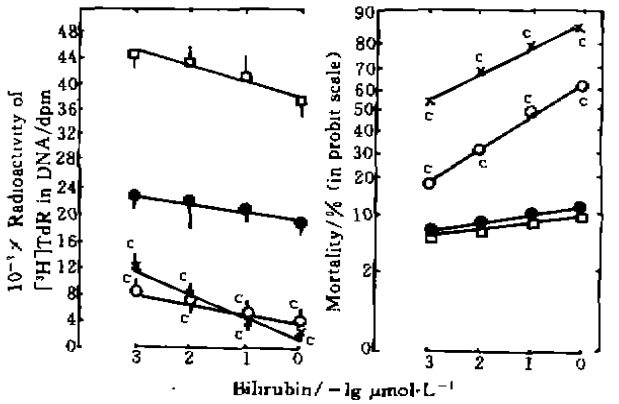

Fig 1. Effect of bilirubin on incorporation of [^{3}H]TdR into DNA and mortality of Hep A cells. 2 h after illumination: (○) light, (●) dark. 24 h after illumination: (×), light, (□) dark. n = 6 experiments, $\bar{x} \pm s$. $^cP<0.01$ *vs* dark.

The inhibition of DNA synthesis of illuminated groups were stronger than those of dark groups ($P<0.01$) and presented a negative concertration dependence. The 1,2-propanediol used as solvent for bilirubin had a little toxicity. The mortality and the inhibition of DNA synthesis were < 0.1 % (Fig 2).

Effects of illuminating time on DNA synthesis and cell mortality The mortality increased with illuminating time from 6.6 % at 1 min up to 63.9 % at 20 min (Fig 2). The DNA synthesis decreased with the illuminating time from 32 × 10^3 dpm at 1 min down to 2.8 × 10^3 dpm (Fig 2).

Effect of bilirubin under room light on DNA synthesis Little difference of DNA synthesis was found between room light-illumination group (1500 − 2000 lx) and dark group ($P>0.05$, Tab 1). It indicated that bilirubin had no phototoxicity under room light, and could be applied in clinic.

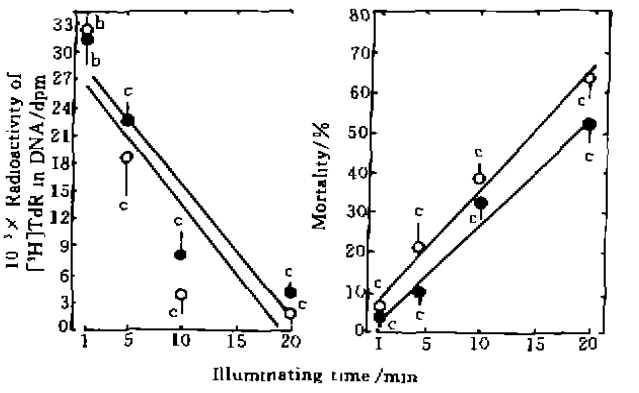

Fig 2. Effect of illumination time on DNA synthesis and mortality of Hep A cells. (○) 3.8 μmol·L^{-1}, (●) 0.38 μmol·L^{-1}. n = 6 experiments, $\bar{x} \pm s$. $^bP<0.05$, $^cP<0.01$ *vs* control.

Tab 1. Effect of bilirubin under room light on DNA synthesis of ascitic hepatoma cells. n = 6 experiments, $\bar{x} \pm s$. $^aP>0.05$ *vs* dark.

Illuminating time/h	Bilirubin/ μmol·L^{-1}	10^{-3} × Radioactivity of [^{3}H] fdR in DNA/dpm Light	Dark
1	3.8	12.9 ± 0.9^a	13.4 ± 3.3
	0.38	14.1 ± 6.7^a	16.3 ± 4.1
3	3.8	16.0 ± 7.5^a	19.1 ± 4.8
	0.38	22.5 ± 13.9^a	30.1 ± 2.1

The mechanism of bilirubin photosensitization Superoxide dismutase (SOD, scavenger of superoxide, O_2^-), mannitol and sodium formate[4-6] (scavengers of hydroxy radical, OH·) could not protect the cells against the photosensitization of bilirubin, thus the photosensitization of bilirubin had no relation with OH· and O_2^-. But NaN_3[5,6] (scavenger of singlet oxygen, $^1O_2$) and catalase (Cat) can protect cells obviously, so the photosensitization is related to $^1O_2$ and H_2O_2 (Tab 2).

Yellow H_4TiO_5 precipitate was noted when illuminated bilirubin (38 μmol·L^{-1}) was mixed with $TiCl_4$ and $NH_3 \cdot H_2O$, while no precipitate was seen

164-166

Tab 2. Effect of free radical scavengers on photosensitization of DNA synthesis in ascitic hepatoma cells by bilirubin. $n = 6$ **experiments.** $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* **dark.**

Scavengers/mg·L^{-1}	$10^{-3}\times$ Radioactivity of [^{3}H]TdR in DNA/dpm			
	2 h after illumination		24 h after illumination	
	Bilirubin/mg·L^{-1}			
	58.4	5.84	58.4	5.84
Control	14.3 ± 0.2	16.5 ± 0.7	13.2 ± 1.2	14.7 ± 0.5
Catalase (38.4)	31.5 ± 2.6^c	38.3 ± 2.3^c	43.4 ± 4.4^c	45.3 ± 3.5^c
Inactive Cat (38.4)	15.7 ± 2.8^a	14.9 ± 3.3^a	15.8 ± 2.3^a	17.6 ± 4.4^a
SOD (74.1)	9.7 ± 5.1^a	12.0 ± 8.0^a	17.2 ± 1.9^a	14.3 ± 8.2^a
Inactive SOD (74.1)	9.3 ± 2.8^a	14.7 ± 0.7^a	12.5 ± 1.0^a	12.2 ± 0.7^a
Mannitol (67.4)	12.3 ± 5.8^a	14.8 ± 3.4^a	18.5 ± 1.8^a	17.2 ± 1.2^a
Sodium formate (37.8)	14.2 ± 2.2^a	12.1 ± 6.4^a	11.5 ± 4.5^a	12.2 ± 0.7^a
NaN_3 (24.1)	30.8 ± 6.1^c	43.5 ± 2.1^c	53.4 ± 5.1^c	31.3 ± 6.4^c

if bilirubin had not been illuminated or Cat was added after illumination. These results proved that H_2O_2 *was involved in photosensitization of bilirubin.*

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胆红素对腹水型肝癌细胞生长及 DNA 合成的光敏作用

周秀芳，陈富裕，曾肇肿，郑荣梁

（兰州大学生物系，兰州 730000，中国）

关键词 胆红素；光敏化剂；肝细胞瘤；肿瘤脱氧核糖核酸；自由基

目的：观察胆红素光敏反应对腹水型肝癌(Hep A)细胞 DNA 的影响及其机理。**方法**：细胞经 1.0×10^5 lx 照光 10 min 后加脱氧[^{3}H]胸苷，测 DNA 的合成。细胞用 0.5 % 台盼兰染色后计数。**结果**：胆红素光敏反应使细胞死亡率增加；DNA 合成明显受到抑制（$P<0.01$）；且随浓度的增加和照光时间的延长而加剧。在自然光照下，照光组与避光组 DNA 合成没有明显区别（$P>0.05$）。**结论**：胆红素光敏反应对 Hep A 细胞有明显的杀伤作用；自然光照组不产生光敏反应；光敏反应的产生与 $^1O_2$ 和 H_2O_2 密切相关，而与 $OH\cdot$ 和 O_2^- 无关。

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