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Analysis of the cell cycle of fibroblasts derived from the LEC rat after X-irradiation

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Abstract

The LEC rat is reported to exhibit hypersensitivity to X-irradiation, deficiency in DNA double-strand break repair, and radio-resistant DNA synthesis. This character of the LEC rat has been thought to be due to abnormal G1 arrest in cells after X-irradiation. In this report, we re-investigated the effect of X-irradiation on the cell cycle in primary-cultured fibroblasts. Primary-cultured fibroblasts derived from LEC and BN rats were exposed to 4 Gy of X-ray and their cell cycle analysis was performed with a flow cytometer. Fibroblasts derived from both rats showed normal response of the cell cycle, indicating the arrest at both G1- and G2/M-phase and no difference in the cell cycle population between fibroblasts derived from both rats. In contrast, when the same analysis was performed using the cell line, L7 and W8, which had been established from the lung fibroblasts of LEC and control WKAH rats, respectively, by immortalizing with SV40 T-antigen, L7 cells but not W8 cells showed impaired G1 arrest and abnormal cell cycle. These results suggest that fibroblasts derived from LEC rats possess the normal cell cycle response after X-irradiation, if they are kept naive as not immortalized with SV40 T-antigen.

Key Words : Cell cycle, Fibroblast, LEC rat, X-irradiation, X-ray hypersensitivity

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Introduction

The LEC rat was established from closed colony of Long-Evans rats as a mutant exhibiting fulminant hepatic disorder^{8,9}. It was also shown that the LEC rat was defective in differentiation of T cells in the thymus². Besides these interesting mutations, the LEC rat was reported to be sensitive to both ionizing radiation and DNA-damaging agents^{5,7}. Quantitative trait locus (QTL) analysis showed that radio-sensitive phenotype was controlled by multiple genetic loci, including a main QTL, *xhs1*, located on chromosome (Chr) 4¹. Further, this phenotype was shown to be due to the impaired repair of DNA double-strand breaks after X-irradiation⁵. Thus, the LEC rat is a useful strain for the study of radiation biology.

Hayashi et al. reported that X-irradiation induced abnormal G1 arrest⁶ and abnormal accumulation of G2/M-phase cells⁴, causing the radio-resistant DNA synthesis in the LEC rat-derived fibroblast cell line that had been immortalized with SV40 T-antigen^{3,6}. In this report, we re-examined the effect of X-irradiation on the cell cycle progression in primary-cultured fibroblasts prepared from the abdominal skin of LEC and control BN rats. We showed that the response of the cell cycle to X-irradiation in fibroblasts derived from LEC rats was normal, and there was no difference in the cell cycle in between fibroblasts derived from LEC and control rats after X-irradiation.

Materials and Methods

Rats

LEC/Ncu and BN/Sea rats were maintained in the animal facility of Nagoya City University Medical School with specific pathogen-free conditions. Pathogenic microorganisms routinely monitored were as fol-

lows; Sendai virus, Sialodacryoadenitis virus, Hanta virus, *Bordetella bronchiseptica*, *Corynebacterium kutscheri*, *Mycoplasma pulmonis*, *Clostridium piliforme*, *Pasteurella pneumotropica*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Giardia muris*, *Trichomonads* spp., and *Syphacia* spp.. Other infectious diseases were recognized free by routine diagnosis by an attendant Veterinarian. The animal rooms were kept at $23 \pm 2^\circ\text{C}$ and $50 \pm 10\%$ humidity under a 12-h light-dark cycle. Rats were housed in polycarbonate cages with sterilized wood chip bedding. A commercially formulated standard diet (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and autoclaved water were provided *ad libitum*. Research was conducted according to the Guideline for the Care and Use of Laboratory Animals of Nagoya City University Medical School. Experimental protocol was approved by the Institutional Animal Care and Use Committee of Nagoya City University Medical School.

Cell culture

The primary culture of fibroblasts was prepared from abdominal skin of LEC and BN rats at 5 weeks of age. The skin samples (1 cm²) were sterilely dissected from rats received euthanasia with inhalation of excessive CO₂. The LEC and WKAH rat-derived fibroblast cell lines, L7 and W8, respectively, which had been established from fibroblasts in lungs by immortalizing with SV40 T-antigen, were kindly gifted from Dr. Masanobu Hayashi, Department of Veterinary Radiology, School of Veterinary Medicine, Rakuno Gakuen University³. Cells were cultured in Dulbecco's modified Eagle medium containing 10% fetal calf serum at 37°C in an atmosphere containing 5% CO₂.

X-irradiation

X-irradiation was carried out at a dose rate of 0.14 Gy/min using a M-80WE X-ray generator (Softex, Ebina, Japan) operating at 80 kVp and 10 mA with 0.1 mm Cu filter.

Colony formation assay

Exponentially growing cells were collected by trypsinization and 500 cells were plated on 60-mm plastic tissue culture dishes 24 h before X-irradiation. The cells were incubated for 10 days after X-irradiation, and the dishes were methanol-fixed and stained with Giemsa. Colonies containing more than 50 cells were counted as survivors.

Cell cycle analysis

Cells were collected from the culture with trypsinization and fixed with 70% ethanol. Fixed cells were centrifuged, treated with 1 mg/ml RNase, and stained with 50 μ g/ml propidium iodide (PI). Stained cells were analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, USA). In order to analyze S-phase cells in detail, cells were

treated with 10 μ g/ml bromodeoxyuridine (BrdU) (Sigma, St. Louis, USA) for 1 h at 37°C and then, fixed with 70% ethanol. The cells were centrifuged, resuspended in 4N HCl, and incubated for 20 min at room temperature. After neutralizing the samples with 0.1M sodium borate, the cells were washed with phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA), stained with anti-BrdU antibody (PharMingen, San Diego, USA), washed with PBS containing 0.5% BSA and 0.5% Tween-20, and then labelled with polyclonal anti-mouse IgG conjugated with fluorescein-5-isothiocyanate (FITC) (PharMingen). Subsequently, the cells were treated with 1 mg/ml RNase and stained with 50 μ g/ml PI. Stained cells were analyzed with a FACScan flow cytometer (Becton Dickinson).

Results

To investigate the sensitivity to the X-irradiation of primary-cultured fibroblasts derived from LEC and BN rats, the cell survival after X-irradiation was determined by colony

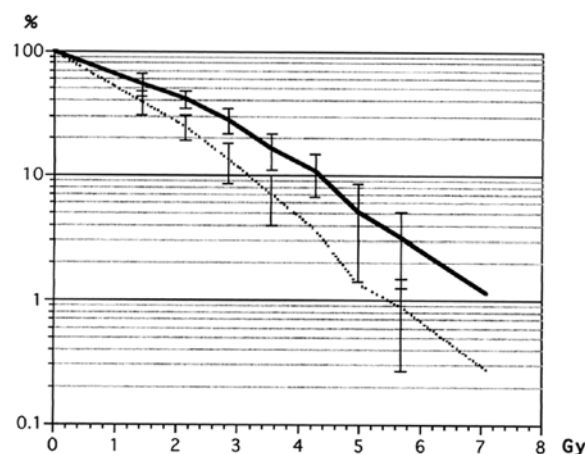


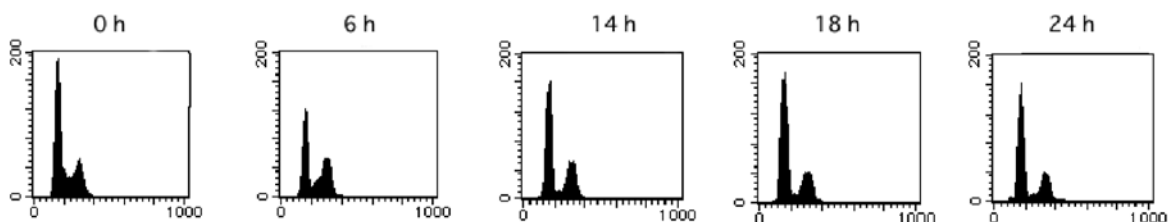
Fig. 1. Colony formation assay of primary-cultured fibroblasts derived from LEC and BN rats after X-irradiation with various doses. The number of colonies was counted at 10 days after X-irradiation. Solid and dashed lines indicate the data from BN and LEC rat-derived fibroblasts, respectively. The data were expressed as means \pm SD.

formation assay. The X-irradiation dose-response curve is shown in Fig. 1. The fibroblasts derived from LEC rats were more sensitive than those of BN rats. Since the dose showing significant difference was 4 Gy, we used this dose in the cell cycle analysis after X-irradiation.

After the primary-cultured fibroblasts derived from LEC and BN rats were exposed to 4 Gy of X-irradiation, the cells were incubated at 37°C for 0-24 h, and their cell cycle distribution was determined by a flow cytometer after PI staining. As shown in Fig. 2 and Table 1, the population of S-phase cells to the total cells decreased from 31.0 to 8.1% in LEC rats, and from 29.8 to 11.0% in BN rats with incubation time after X-irradiation. The population of G1-phase cells once decreased at 6 h post-irradiation from 53.1 to 46.5% in LEC rats, and from 57.3 to 44.9% in BN rats, and then conversely increased with incuba-

tion time to become 66.9 and 69.2% at 24 h post-irradiation in LEC and BN rats, respectively. The population of G2/M-phase cells once increased at 6 h post-irradiation from 15.9 to 38.0% in LEC rats, and from 13.0 to 40.8% in BN rats, and then conversely decreased with incubation time to become 25.1 and 19.8% at 24 h post-irradiation in LEC and BN rats, respectively. Further, the decrease of S-phase cells was confirmed by the BrdU incorporation analysis (Fig. 3). As shown in Fig. 3, BrdU-labelled cells certainly decreased at both 12 and 24 h post-irradiation in both LEC and BN rats. These results indicate that the checkpoint response in the cell cycle occurred as follows. 1) After X-irradiation, the G2/M arrest occurred at the early period (at 6 h post-irradiation). 2) The G1 arrest followed the G2/M arrest and sustained from 6 to 24 h post-irradiation. 3) These normal checkpoint response occurred

LEC rat fibroblast cells



BN rat fibroblast cells

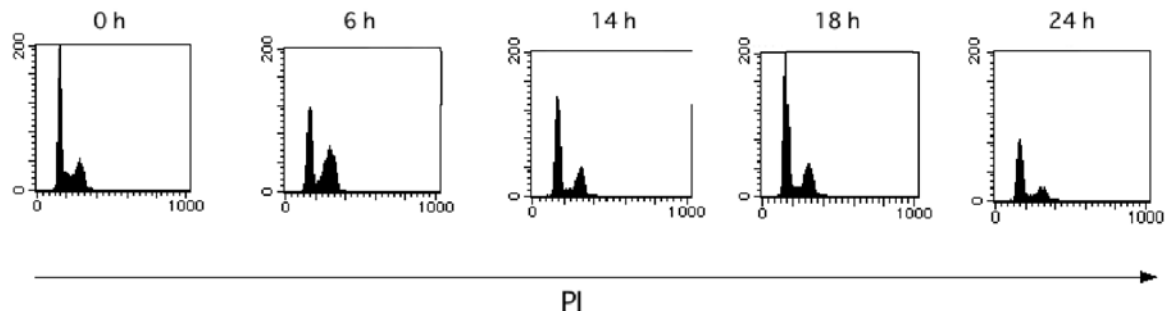


Fig. 2. Cell cycle analysis of primary-cultured fibroblasts derived from LEC and BN rats after X-irradiation. Cells were X-irradiated at 4 Gy and then incubated at 37°C for 0, 6, 14, 18, and 24 h. The cell cycle analysis was performed with a flow cytometer at each incubation time.

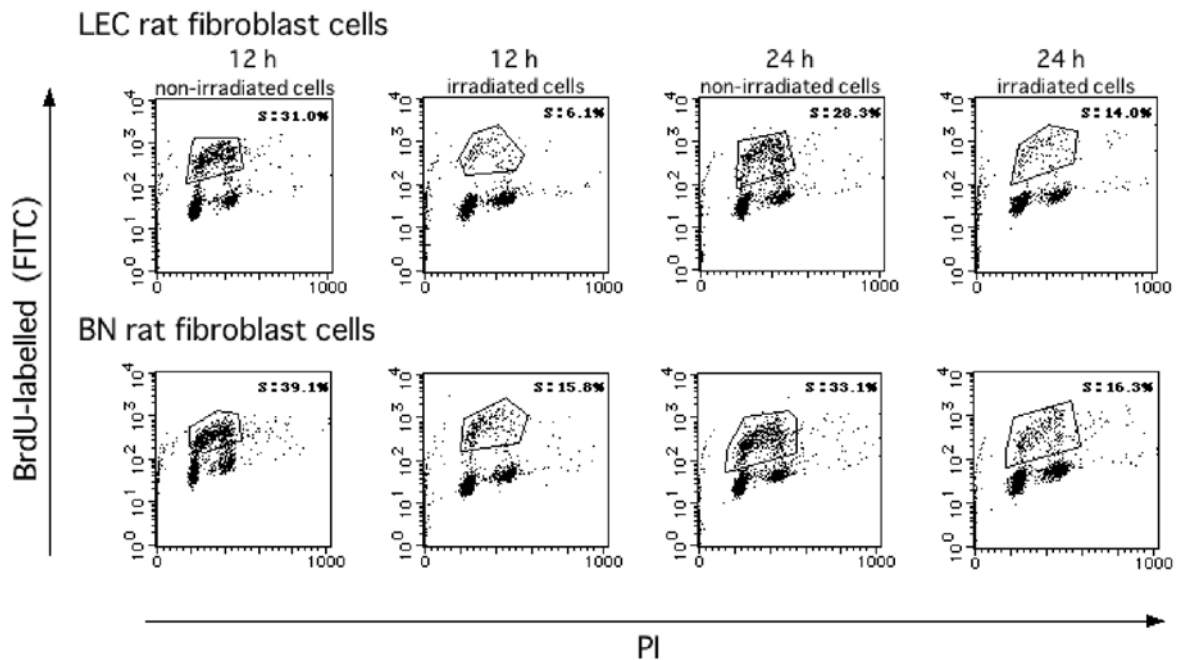


Fig. 3. S-phase cell analysis with BrdU incorporation of primary-cultured fibroblasts derived from LEC and BN rats after X-irradiation. Cells were X-irradiated at 4 Gy and incubated at 37°C for 12 and 24 h. Subsequently, at each time, the cells were treated with BrdU at 37°C for 1 h, and then, analysis was performed with a flow cytometer.

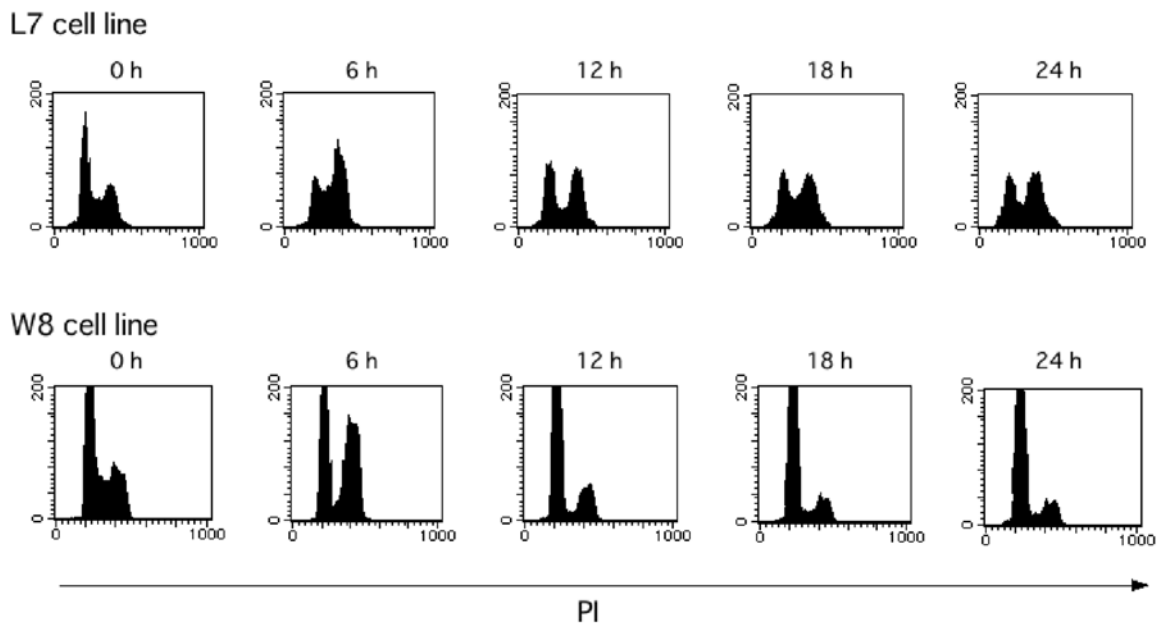


Fig. 4. Cell cycle analysis of fibroblast cell lines, L7 and W8, derived from LEC and WKAH rats, respectively, after X-irradiation. Cells were X-irradiated at 4 Gy and then incubated at 37°C for 0, 6, 12, 18, and 24 h. The cell cycle analysis was performed with a flow cytometer at each incubation time.

Table 1. Cell population in the cell cycle of fibroblasts after X-irradiation

		Cell population (%)		
		G0 & G1	S	G2 & M
Primary culture				
LEC	0h	53.1	31.0	15.9
	6h	46.5	15.5	38.0
	14h	62.1	6.0	31.8
	18h	66.2	7.5	26.3
	24h	66.9	8.1	25.1
BN	0h	57.3	29.8	13.0
	6h	44.9	14.3	40.8
	14h	66.9	9.9	23.2
	18h	66.2	13.6	20.3
	24h	69.2	11.0	19.8
Cell line				
L7	0h	47.5	19.5	32.5
	6h	23.7	31.2	44.8
	12h	36.0	14.0	49.2
	18h	29.6	21.4	45.1
	24h	30.2	18.1	45.2
W8	0h	58.7	16.2	24.0
	6h	44.9	6.6	47.8
	12h	80.8	3.0	15.5
	18h	86.2	2.9	9.1
	24h	84.7	3.5	11.2

in fibroblasts derived from both LEC and BN rats without difference.

Inconsistent with our results, previous papers reported that fibroblast cell line derived from LEC rats showed abnormal checkpoints of both G1 and G2/M arrests^{4,6}. To check if the discrepancy is attributed to the experimental conditions or to the cell type of fibroblasts, primary-cultured cells vs. cell line immortalized with SV40 T antigen, we analyzed the cell cycle using the same cell line as used in the previous reports^{4,6} (Fig. 4 and Table 1). S-phase of the L7 cells derived from LEC rats did not decrease so efficiently after X-irradiation. G1-phase of L7 cells did not increase after X-irradiation but rather decreased. In contrast, G2/M-phase of L7 cells increased slightly. These data suggest that checkpoints in the cell cycle of L7 cells are severely impaired, although G2/M arrest remains relatively normal. On the other hand, S-phase of W8 cells derived from normal rat

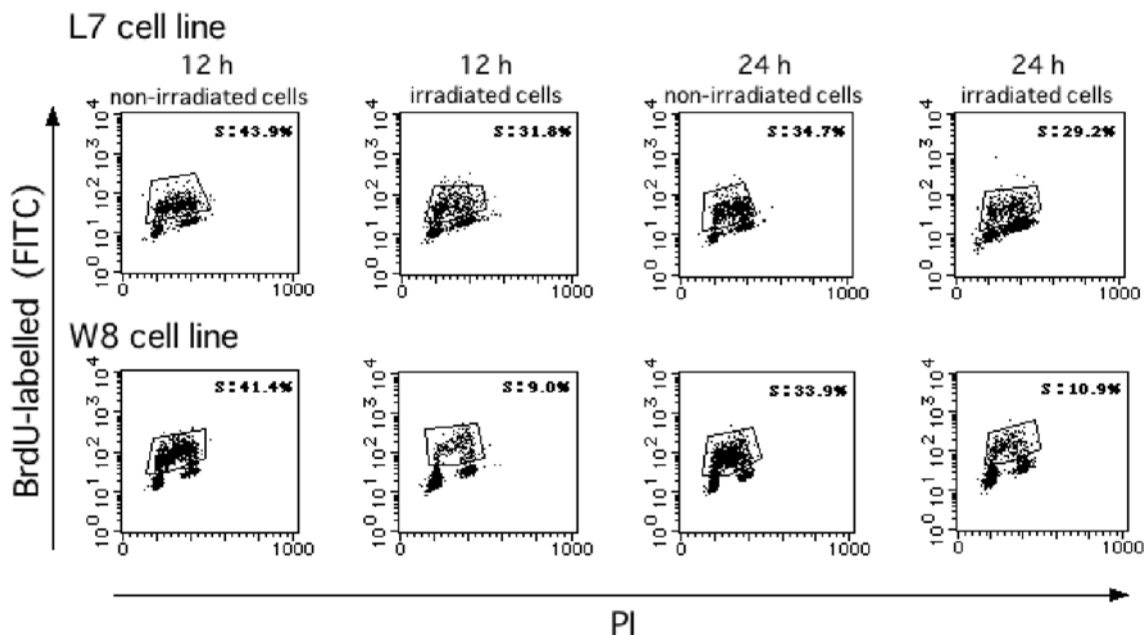


Fig. 5. S-phase cell analysis with BrdU incorporation of L7 and W8 cell lines after X-irradiation. Cells were X-irradiated at 4 Gy and incubated at 37°C for 12 and 24 h. Subsequently, at each time, the cells were treated with BrdU at 37°C for 1 h, and then, analysis was performed with a flow cytometer.

strain, WKAH, decreased quickly after X-irradiation. G1-phase increased from 58.7 to 84.7-86.2% at 18-24 h post-irradiation. G2/M-phase increased from 24.0% to 47.8% at 6 h post-irradiation, and then decreased to 11.2% at 24 h post-irradiation. These data suggest that checkpoints in the cell cycle of W8 cells were essentially the same as that of BN rat-derived primary-cultured fibroblasts, namely, the G2 arrest occurred at 6 h post-irradiation and followed by the G1 arrest. To make sure the change of the S-phase population, BrdU incorporation analysis was performed (Fig. 5). S-phase of L7 cells did not decrease efficiently, whereas that of W8 cells decreased quickly from 41.4 to 9.0% at 6 h post-irradiation and sustained in the low level by 24 h post-irradiation.

Discussion

It has been reported that fibroblasts derived from LEC rats show abnormal G1 arrest⁶⁾ and abnormal accumulation of G2/M-phase cells⁴⁾ after X-irradiation and that these abnormal checkpoints after X-irradiation are thought to be one of the causes for the hypersensitivity to X-irradiation in LEC rats. However, in our data with primary-cultured fibroblasts derived from LEC and BN rats, there was no difference of the cell cycle after X-irradiation between them. After X-irradiation, the G2/M arrest occurred at 6 h post-irradiation followed by the G1 arrest in primary-cultured fibroblasts derived from both LEC and BN rats. By performing the cell cycle analysis using the cell line, L7 and W8 cells, which had been used in the previous reports^{4,6)}, we could show that abnormal cell cycle in L7 cells, suggesting that discrepancy between our and previous results are attributed not to the experimental conditions but to the cell types. Thus, L7 cells were established from fibroblasts derived from LEC rat lung by

immortalizing with SV40 T-antigen. SV40 T-antigen is known to suppress p53 proteins, which play a key role in the cell cycle arrest. Hayashi et al. showed that the expression level of the p53 protein increased in W8 cells, while no significant increase of p53 was observed in L7 cells after X-irradiation⁶⁾. This result indicates that the expression of p53 in L7 cells may be suppressed by SV40 T-antigen. Thus, we should pay attention to the overestimation of the results using the fibroblast cell line immortalized with SV40 T-antigen. In this paper, we showed normal cell cycle checkpoints in the LEC rat-derived fibroblasts, indicating that the X-ray hypersensitivity of the LEC rat is due to unknown cause rather than the abnormal checkpoints after X-irradiation. We have recently mapped the *xhs1*, which is a main QTL responsible for the X-ray hypersensitivity of the LEC rat, to the middle region of Chr 4¹⁾. An as-yet-unidentified gene in this region seems to be responsible for the X-ray hypersensitivity of the LEC rat. Identification of the gene responsible for the X-ray hypersensitivity is now in progress.

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