Circadian rhythms of proliferation events in two mouse carcinomas

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We studied the index of DNA synthesis (DNAs) of two cellular carcinomas: the hepatocellular ES12a and the mammary TN60 of mice, throughout one circadian cycle. In the results, we observed that both tumors have circadian rhythms (CRs), but the peaks of DNAs vary. Besides, the mean of DNAs along 24 h shows significative differences, the TN60 has higher values than the ES12a. These observed CR in the DNAs index in both carcinomas mean that, at least in partly, the proliferation of cancer cells can be regulated by endocrine factor as it normally occurs in ordinary cells. The big problem we can find for the chronopharmacology is that it is impossible to know in advance the rate of proliferation of each tumor.

\textbf{Keywords:} carcinoma; circadian rhythms; DNA synthesis; mice

1. Introduction

Cell Cycle length may vary, in numerous cell populations, in response to specific conditions associated with different stages of the organism’s life span. It is regulated by growth factors and other signaling molecules that stimulate or inhibit cellular proliferation (García et al. 2001). In mammals, circadian rhythms (CRs) are generated by a central clock in the suprachiasmatic nucleus located in the hypothalamus that constantly synchronize with environmental cues via circadian input pathways and controls the peripheral clocks through circadian output pathways (Reppert & Weaver 2002; Buijs et al. 2006).

The circadian system is perturbed by exposure to light at night with commensurate suppression of melatonin production and dysregulation of circadian genes that have been implicated in cancer development (Takahashi et al. 2008). Shift work that involves circadian disruption is deemed to be a probable carcinogen by the International Agency for Research on cancer and epidemiologic studies. It has been demonstrated that CR disruption increases the risk of breast, colon, prostate, lung, ovarian, and hepatocellular carcinoma (Davis et al. 2001; Straif et al. 2007; Baan et al. 2009).

Tumor growth and evolution are a complex phenomenon controlled by an intricate pattern of competing processes (Perez de Castro et al. 2007). Previous studies have demonstrated that many cellular neoplastic populations like some hepatomas and hepatocellular carcinomas show circadian variations in the synthesis of DNA (DNAs) (Nash & Echave Llanos 1971; García et al. 2006). In some cases, the observed rhythm was
similar to that of the original cell population (Barbeito et al. 1995), but, some undifferen-
tiated hepatocellular carcinomas did not show any mitotic CR (Moreno et al. 1985). Moreover, diurnal levels of pituitary gonadotropins is of self-evident interest in the biology of endocrine-related tumors such as breast and prostate cancer (Kelleher et al. 2014).

There are many techniques to analyze the different phases of the cell cycle. Estimation of S-phase index (SI) can be obtained by detection of labeled nuclei by immunohistochemical methods like bromodeoxyuridine (Brdu), a thymidine analog, as an indicator of cellular proliferation (Thomson et al. 1999).

The following experiments were designed in order to study and compare the DNAs index of two different cellular carcinomas of adult male mice: the hepatocellular ES12a and the mammary TN60, throughout one complete circadian cycle.

2. Materials and methods

2.1. Animals

For these experiments, we used adult male C3H/S-strain mice. Conditions concerning animal management fully respected the policy and mandates of the Guide for the Care and Use of Laboratory Animal Research of the National Research Council. They were subjected to the following standardization conditions: water and food available ad libi-
tum, ambient temperature maintained at 22 ± 2 °C, alternating light and dark periods restricted to 12 h each with illumination by fluorescent lamps beginning at 06:00 h.

2.2. Tumor-bearing animals

After an appropriate period of synchronization (15 days), about 70 mg of the C3H/ S-histocompatible mammary or hepatocellular carcinomas was grafted separately into the subcutaneous tissue of each animal’s flank. These carcinomas were maintained by subcutaneous serial transplant in male mice. The graft-bearing animals, subsequently divided into lots of 5–8 mice each, were then housed for further two weeks under standardization conditions before the lots were separated into the following experimental-protocol groups.

2.3. Experimental groups

The animals were divided in two experimental groups: Group I, mice bearing the ES12a hepatocellular carcinoma, and Group II, mice bearing the TN60 mammary carcinoma. When the tumors were about 2 ± 0.5 cm of diameters, every group was divided into 6 lots of 4/8 animals each. Every lot was sacrificed every 4 h starting at 00:00 h throughout one complete circadian cycle. In the necropsy of the animals, we extracted the solid tumor and these were processed as we mentioned before.

2.4. S-phase index

To determine the index of DNAs of tumor cells, we used immunohistochemistry. One hour before being killed, all animals, received an intraperitoneal injection of 50 mg/kg of 5-bromodeoxyuridine (Sigma, St. Louis, USA). Samples of apparently nonnecrotic tumor tissue were excised and fixed in 10% buffered formalin for 24 h and embedded
in paraffin. Sections (5 μm) were placed on silanized slides, dried overnight, deparaffinized in xylene, rehydrated through graded alcohols and washed in Tris-buffered saline (TBS) at pH 7.4. Endogenous peroxidase was blocked with 3% H₂O₂ for 10 min. Antigen retrieval was achieved by washing the slides in TBS and irradiating them in citrate buffer, pH 6.0 at 750 W for two cycles of 5 min in a microwave oven. After microwaving, the slides were washed in TBS and incubated with primary antibodies (Bu 20a, 1/100, Dako, Carpinteria, CA, USA) for 1 h at room temperature. Envision was used as a detection system with 3’3-diaminobenzidine (Sigma, St. Louis, MO) as the chromogen. The sections were lightly counterstained with Mayer’s hematoxylin.

The specimens were then examined microscopically under an oil-immersion objective (at 1.500 X) in order to score the total number of labeled nuclei among a minimum of 3000 nuclei. From these data, the SI was calculated and expressed as the number of labeled nuclei per 1000 nuclei.

2.5. Statistical analysis
The results are expressed as means ± SE. For evaluating the statistical significance of differences among the means, we used first the Anova and Student–Newmann–Keuls Multiple Comparisons Test.

3. Results
As we can see on Table 1 and Figure 1, both tumors have CRs in their DNAs, and the minimum values were observed at the same time (between 08:00 and 12:00 h), but the peak of DNAs vary because in the ES12a is at 00:00 h (156.5 ± 28.3) and in the TN60 is at 20:00 h (190.4 ± 14.4).

Furthermore, the mean of DNAs along 24 h have significative differences (p = 0.007) between ES12a and TN60. The last one, the mammary, has higher values than the hepatic carcinoma.

In Figure 1, we could observe that in ES12a hepatocellular carcinoma the values between the maximum and minimum of their ADNs have much more significative differences (p < 0.001) than also significant differences observed between the maximum and minimum values in TN60 mammary carcinoma (p < 0.05).

Table 1. DNAs values along a circadian time span for ES12a and TN60 carcinoma.

| HD    | ES12a X ± ES (n) | TN60 X ± ES (n) |
|-------|-----------------|-----------------|
| 00:00 | 156.5 ± 28.3 (8) | 107.6 ± 9.5 (4) |
| 04:00 | 102.3 ± 21.6 (6) | 115.3 ± 16.9 (6) |
| 08:00 | 22.0 ± 7.4 (5) | 101.4 ± 21.9 (5) |
| 12:00 | 21.9 ± 14.6 (5) | 161.6 ± 13.1 (6) |
| 16:00 | 28.3 ± 11.9 (6) | 173.0 ± 17.7 (6) |
| 20:00 | 86.9 ± 17.7 (7) | 190.4 ± 14.4 (5) |
| X     | 69.6 ± 20.7 (37) | 144.8 ± 9.7 (32) | (p = 0.0073) |

Note: HD: hour of day; X: mean; ES: standard error; n: number of animals; p: probability.
4. Discussion

The circadian variation in human normal tissues has been described since 1938 (Cooper 1938), but we could find very few reports on CR of DNAs in human tumor type, and the majority of these studies were made without check a full day. However, we can mention a report that analyze the cellular proliferation in human breast cancers, in which they found changes during each estrous cycle and each season throughout each year (Oh et al. 2001).

In this study, we observed CR in the DNAs index in both murine carcinomas: the hepatocellular ES12a and the mammary TN60, this means that, at least in a partly, the proliferation of cancer cells can be regulated by endocrine factor as it occurs in ordinary cells. As it can be observed in previous works and it has been demonstrated in this research, it is true that the majority of solid tumors have rhythms in their proliferation activity. The differences in the average in the proliferation of tumors along a day are probably due a numerous factors: as the original tissue of the tumor, the degree of malignancy, degree of differentiation of cell type, GF, and others mitotic controls. We can mention a lot of GF involved in control cellular proliferation, including HGF, EGF, TGFβ, IGF, etc. (Akiel et al. 2014). Probably, one or more of these are responsible for regulating the CR of proliferation observed in this study.

The big problem that we can find is that it is impossible to know in advance the rate of proliferation of each tumor and obviously this study cannot be applied to human patient with cancer. We could try to know the rate of proliferation of specific neoplastic cell performing a cell culture of them, but we could not be sure that the results found in culture are the real proliferation rate in vivo; as Loning and Kettner said: ‘The clock-controlled genes usually do not share overlapping expression patterns between tissues, suggesting a key role for the circadian clock in controlling tissue-specific function in vivo’ (2013).

All these preliminary considerations are nothing more than speculation since we are far from knowing at least the CR of proliferation of the most common malignancies in
man. Perhaps if we could correlate our results in mice (with nocturnal habits) and we can transpose this to humans (with diurnal habits), we can suppose that the most important DNAs activity probably appears during diurnal moment for the implementation of anticancer drugs, to increase its positive effects. In addition, we must mention that “The changes in lifestyle are coupled with a significant increased in the risk of diseases in all aspects of human health, including cancer” (Loning & Kettner 2013).

If we knew the proliferative rate of each specific kind of tumor we could apply the principle of the chronopharmacology, which propose that the CRs can affect the effects of drugs, improving their benefits or increasing its adverse effects (Nakahata et al. 2007). This would lead us to a significant improvement in the treatment and control of the cancer. Therefore, to increase the maximum efficiency of attack against tumor and protect normal host tissue are the biggest challenges for successful anticancer treatment (Loning & Kettner 2013).

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No potential conflict of interest was reported by the authors.

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