# The time of tumor cell division and death depends on the site of growth

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**Abstract.** We show here, for the first time, in two very different murine tumors, a mammary one (ectoderm) and a lung one (endoderm), that: tumors have day/night differences of spontaneous apoptosis additional to the well-known circadian rhythm of mitosis. The times of maximal and minimal mitosis and apoptosis changed for a tumor cell line when growing in different organs (as metastasis) or anatomical sites. Both tumor lines, have identical circadian curves when growing in a specific organ or anatomical site. The peaks of apoptosis match with the valleys of mitosis and vice versa.

## Introduction

It is well-known that, in mammals, the day/night rhythm influences the activity of most physiological systems, such as immune, reproductive, gastrointestinal, cardiovascular, endocrine, nervous and other systems (1-3). Furthermore, it was demonstrated that cell division occurs in different organs preferentially at different hours of the day; i.e., there is a peak of mitotic figures at noon in murine liver, at 03:00 a.m. in human gastric mucosa, at midnight in human bone marrow and skin (4-6).

Primary human and murine tumors also show a circadian rhythm for mitosis, being the time of the maximal proliferative activity different according to the type of tumor (4,7,8). Thus, the peak of mitotic figures in a murine gastric cancer is at noon, in some murine sarcomas at 01:00 a.m., in human mammary adenocarcinoma in the early afternoon, in human uterine cervix and in human skin tumors around midnight (4).

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These differences are the bases of Chronopharmacology in Oncology, i.e. the increase in the doses of drugs during the time of the day when they are more useful because more tumor cells are in the phase of the cell cycle that this drug works, while less of normal cells (from bone marrow, gut, skin, etc.) are in the same phase, thus increasing effectivity and decreasing toxicity (9,10).

In a tumor, many cells proliferate while at the same time some cells die either by necrosis or apoptosis. As a result of a balance of these processes the tumor mass may increase, decrease or remain stable (11).

We tried to answer the questions: i) Does tumor cell death present circadian variations in the manner of tumor cell division? ii) Does a tumor cell line maintain its circadian rhythms when growing in different organs or anatomical sites?

## Materials and methods

*Tumors.* PO7 is a poorly differentiated and highly metastatic lung adenocarcinoma. M3 is a semidifferentiated moderately metastatic mammary adenocarcinoma. Both tumors arose spontaneously in female Balb/c mice from our colony and are maintained by subcutaneous transplants in female Balb/c mice. Both tumors grow with the same behaviour in males than in females.

*Mice.* Three-month old brother/sister mated syngeneic male Balb/c mice from our animal facility were inoculated subcutaneously with tumors with the aid of trocars.

In some groups of mice, a small tumor piece was inoculated into spleen with a thin trocar, or deposited into the peritoneal cavity, in a surgical procedure, opening the abdominal wall, under Embutal anesthesia.

Animals care was provided according to the specific international laws and in full compliance with regulations for protection of animals.

*Time of sacrifice.* Sacrifice was done at 6:00 a.m., 12:00 h (noon), 6:00 p.m. or 24:00 h (midnight). Light on at 7:00 a.m. and off at 7:00 p.m.

Mice were sacrificed between 17 and 25 days after transplant, depending on the site of growth and the tumor,

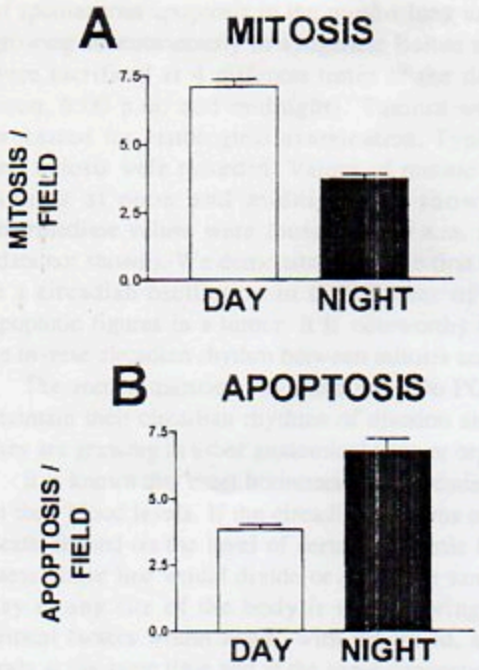


Figure 1. Circadian rhythm of mitosis (A) and apoptosis (B) in PO7 tumors growing subcutaneously. □, day (noon); ■, night (midnight). The mean of the 20 fields from a tumor became 1 data, then each mouse brings 1 datum from mitosis and 1 from apoptosis. The S.D. of each slide were always under the 15% of the mean. Day vs. night were always $p<0.001$ (Student's t-test) (n=5-20).

when they were in the exponential phase of growth, and large enough to obtain tumor tissue for microscopic analysis.

*Histological analysis.* For the morphological quantification of mitosis and apoptosis, tumor tissues were fixed in 4% buffered formaline and paraffin embedded, and 5 μm thick slides were stained with hematoxilin and eosine. Twenty fields of 500X from each slide were analyzed using a Carl Zeiss III microscope, and the number of mitosis and apoptosis per field were recorded.

The criteria used in identifying morphologically early apoptosis were: tumor cells isolated from its neighbors, with shrinkage and margination and clumping of nuclear chromatin (12). Only the very typical apoptotic figures were recorded.

Day/night differences in the number of apoptotic cells were confirmed with the TUNEL technique (Apoptag Plus, Oncor Inc., MD).

## Results

The first question we asked was: Does tumor cell death present circadian variations in the manner of tumor cell division?

Potten has reported previously that there is a slight circadian oscillation in the number of spontaneous apoptotic figures in normal murine small intestine (13). We studied the number

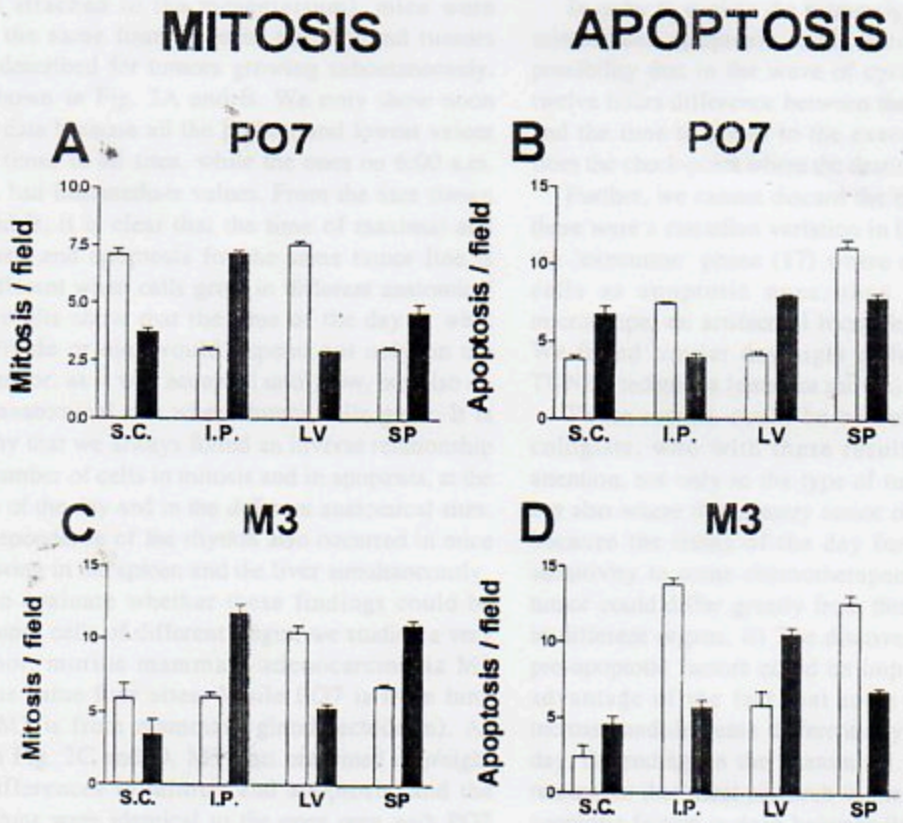


Figure 2. Tissue dependent circadian variations of cell division (A and C) and spontaneous apoptosis (B and D). □, day (noon); ■, night (midnight). A and B, PO7 tumor; C and D, M3 tumor. Liver metastasis (LV) and tumors in spleen (SP) came from the same mice. Each mouse brings one datum for mitosis and one for apoptosis when growing subcutaneously (SC) or in peritoneal cavity (IP), but two (spleen and liver) when transplanted into the spleen. Data recording as in Fig. 1. Day vs. night were always $p<0.001$ (Student's t-test) (n=5-20).

of spontaneous apoptosis in the murine lung tumor PO7 (14) growing subcutaneously in syngeneic Balb/c male mice that were sacrificed at 4 different times of the day (6:00 a.m., noon, 6:00 p.m. and midnight). Tumors were fixed and processed for histological examination. Typical apoptosis and mitosis were recorded. Values of mitotic and apoptotic figures at noon and midnight are shown in Fig. 1. Intermediate values were found at 6:00 a.m. and 6:00 p.m. (data not shown). We demonstrate for the first time that there is a circadian oscillation in the number of spontaneous apoptotic figures in a tumor. It is noteworthy that there was an inverse circadian rhythm between mitosis and apoptosis.

The second question we asked was: Do PO7 tumor cells maintain their circadian rhythms of division and death when they are growing in other anatomical sites or organs?

It is known that most hormones have circadian fluctuations in their blood levels. If the circadian rhythms of division and death depend on the level of certain systemic hormones, the same tumor line would divide or die at the same time of the day in any site of the body it was growing, because all critical factors would reach, with the blood, all sites of the body at the same time and at the same concentration levels. It could also be considered that this rhythm could be modified or driven by the extracellular matrix (ECM) or by growth factors (GF) released by normal cells of the site where the tumor cells grow. Thus, to elucidate the participation of the microenvironment, we inoculated PO7 tumor cells into the spleen or the peritoneal cavity of mice. When tumors in exponential phase of growth reached a critical mass in spleen, liver (as metastasis derived from the spleen) or peritoneum (as solid masses attached to the mesenterium), mice were sacrificed at the same four times of the day and tumors processed as described for tumors growing subcutaneously. Results are shown in Fig. 2A and B. We only show noon and midnight data because all the highest and lowest values were at these times in all sites, while the ones on 6:00 a.m. and 6:00 p.m. had intermediate values. From the data shown in Fig. 2A and B, it is clear that the time of maximal and minimal mitosis and apoptosis for the same tumor line is completely different when cells grow in different anatomical sites. These results show that the time of the day at what tumor cells divide or die, would depend not only on the origin of the tumor, as it was accepted until now, but also on the organ or anatomical site where tumor cells grow. It is also noteworthy that we always found an inverse relationship between the number of cells in mitosis and in apoptosis, at the different times of the day and in the different anatomical sites. This local independence of the rhythm also occurred in mice with cells growing in the spleen and the liver simultaneously.

In order to evaluate whether these findings could be extended to tumor cells of different origin, we studied a very different tumor, murine mammary adenocarcinoma M3 growing at the same four sites. While PO7 is from lung (endoderm), M3 is from mammary gland (ectoderm). As we can see in Fig. 2C and D, M3 also presented day/night significant differences in mitosis and apoptosis, and the circadian rhythms were identical to the ones seen with PO7 in the four sites studied.

## Discussion

We show here that: i) Tumors have day/night variations of spontaneous apoptosis. ii) The times of maximal and minimal mitosis and/or apoptosis change for a tumor cell line when growing in different organs (as metastasis) or anatomical sites. iii) The time of maximal mitosis was always 12 h apart of the time of maximal apoptosis, in any site, in the two very different tumor lines we used. iv) Both tumor lines, a mammary one (M3) and a lung one (PO7) have identical circadian curves in mitosis and in apoptosis when growing in a specific organ or anatomical site.

Do these data suggest that independently of the origin, tumor cells would divide and die according to the circadian rhythm imposed by the anatomical site where they grow? Experiments using other tumor types would be necessary to answer this question. However, in mice with tumors growing in the four sites simultaneously, each site maintained their respective rhythms (data not shown). This means that metastatic cells would divide and die at different times of the day than primary tumor cells.

Moreover, similar results were obtained when we used F-1 (Balb/c x CBA/CAJ) or F-1 (Balb/c x C57Black/6) instead of Balb/c mice (data not shown).

We do not know whether these local circadian rhythm are consequences of: i) Changes in the release or activation of the same or different local growth or pro-apoptotic factors. ii) Changes in the sensitivity of tumor cells to certain factors. iii) Changes in the tumor blood flows (15,16). iv) Different tumor cell subpopulations.

In order to explain the extremely opposite rhythm between mitosis and apoptosis in all sites we cannot discard the possibility that in the wave of cycling cells there is around twelve hours difference between the time to arrive to mitosis and the time to arrive to the execution phase of apoptosis from the check-point where the destiny of the cells is decided.

Further, we cannot discard the theoretical possibility that there were a circadian variation in the duration of the part of the 'execution' phase (17) where most techniques identify cells as apoptotic ones, thus producing, under the microscope, an artifactual increase in the number of them. We found similar day/night differences when using the TUNEL technique (data not shown).

These results could be useful to: i) Chronopharmacologists, who with these results in mind, should pay attention, not only to the type of tumor (as done until now), but also where the primary tumor or metastasis are growing, because the times of the day for maximal and minimal sensitivity to some chemotherapeutical drugs of a primary tumor could differ greatly from those of metastasis growing in different organs. ii) The discovery of new growth and/or pro-apoptotic factors could be improved if researchers take advantage of the fact that some of these factors would increase and decrease differentially at different times of the day, depending on the anatomical site. Moreover, we must remember that most research in the field of growth and pro-apoptotic factors is done between 9:00 a.m. and 6:00 p.m. At these time points several of them could be in very low levels to be discovered. iii) Tumor metastasis growth is the

consequence of the positive balance between the generation of new tumor cell by mitosis and the tumor cell death by apoptosis or necrosis, and organ microenvironment is able to modify several metastatic tumor cell functions (18-20), thus it could be important to study with the goal of modulating the organ microenvironment in such a way that the organ environment could be changed to the conditions observed at the time of the day when less tumor cells divide and more tumor cells die, resulting in a more effective control of metastasis growth and improvement of patient's survival.

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