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Circadian Variation in the Expression of Cell-Cycle Proteins in Human Oral Epithelium

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At the tissue level, there is experimental and clinical data to suggest a cytokinetic coordination of the cell cycle with a greater proportion of cycling cells entering S-phase and mitosis at specific times of the day. The association of certain cell-cycle proteins with defined events in the cell cycle is well established and may be used to study the timing of cell-cycle phases over 24 hours. In this study oral mucosal biopsies were obtained from six normal human volunteers at 4-hour intervals, six times over 24 hours. Using immunohistochemistry, the number of positive cells expressing the proteins p53, cyclin-E, cyclin-A, cyclin-B1, and Ki-67 was determined for each biopsy and expressed as the number of positive cells per mm of basement membrane. We found a statistically significant circadian variation in the nuclear expression of all of these proteins with the high point of expression for p53 at 10:56 hours, cyclin-E at 14:59 hours, cyclin-A at 16:09 hours, cyclin-B1 at 21:13 hours, and Ki-67 at 02:50 hours. The circadian variation in the nuclear expression of cyclins-E (G1/S phase), -A (G2-phase), and -B1 (M-phase) with a normal physiological progression over time suggests a statistically significant circadian variation in oral epithelial cell proliferation. The finding of a circadian variation in the nuclear expression of p53 protein corresponding to late G1 is novel. This information has clinical implications regarding the timing of chemotherapy and radiotherapy. ***(Am J Pathol 1999, 154:613–622)***

Four phases have been defined in the eukaryotic cell division cycle: gap 1 (G1), DNA synthesis (S), gap 2 (G2) and mitosis (M).[1] Passage through the cell cycle is precisely coordinated and controlled by a family of cyclin-dependent kinases (cdk), which are activated by binding to cyclin proteins,[2,3] regulated by phosphorylation,[4] and inhibited by cdk inhibitors.[5,6] The cdks regulate biochemical pathways, or checkpoints, that integrate mitogenic and growth inhibitory signals and monitor chromosome integrity.[7,8] The cyclins play an important regulatory role in this process because they are synthesized and degraded over relatively brief periods of time at sharply defined points of the cell cycle.[9,10] Passage through G1 into S phase is regulated by cyclins D, E, and A and associated kinases, whereas cyclin B1 and associated kinases regulate the G2/M transition.

At the cellular level, each cell must go through the cell cycle in an orderly and controlled fashion where the multiple steps associated with each phase must be successfully completed before progressing to the next. At the tissue level, there is experimental and clinical data to suggest that a similar progression through the cell cycle occurs with a greater proportion of cycling cells in a specific organ entering S-phase and mitosis at specific times of the day.[11] Scheving et al[12] were the first to report a statistically significant circadian rhythm in both the mitotic index and DNA synthesis in mouse duodenal epithelium. In subsequent studies, on different strains of mice, similar observations were made for the epithelium of tongue, esophagus, stomach, jejunum, and rectum.[13] These observations have been confirmed in two clinical studies in which human rectal mucosa biopsies were performed over 24 hours and in a limited study on human oral mucosa.[14–16] An analogous statistically significant circadian variation in bone marrow proliferative activity has been documented in both rodents and humans.[17,18] It is important to note that it has not been demonstrated, by following the same cells throughout the day, whether these daily rhythms result from a wave of progression of the same cells through the cell cycle or if different cells participate in this circadian population dynamic at different times of the day.

How the circadian variation in proliferation at the tissue level relates to the control of the cell cycle and the detection of the different cyclins has not been studied before. Most studies examining the timing of the expression of different cyclin proteins relative to known cell-cycle events have been performed on synchronized cells in

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culture. This methodology does not permit detection of cyclin protein expression with regard to the time of day nor topographically within the normal architecture of the tissue of interest. Furthermore, the perturbations required for cell synchronization lead to growth inhibition and may bias the findings.[19,20]

The predictable association of certain cell-cycle proteins with defined events in the cell cycle may be used to study the timing of cell-cycle phases in normal tissues. We hypothesized that a circadian variation in proliferation would be associated with a circadian variation in the expression of the cell-cycle-associated proteins, cyclin-E, cyclin-A, cyclin-B1, as well as p53 and Ki-67. If this were true, it would provide a better basic understanding of the biology of epithelial cell division and cell-cycle control. Moreover this might have clinical implications for the timing of both chemotherapy and radiotherapy. Here we describe a study to test this hypothesis on oral mucosa biopsies obtained from six normal human volunteers at 4-hour intervals over 24 hours. We studied, by immunohistochemistry, the time of day-dependent variation in the number of cells showing nuclear staining for the proteins cyclins-A, -E, -B1, as well as p53 and Ki-67. Using this experimental system, we were able to detect the nuclear expression of these proteins as a function of time of day in unperturbed, anatomically intact, human epithelium.

Materials and Methods

Subjects and Tissue Handling

The Sunnybrook Health Science Center Ethics Review Board approved the study protocol, and all subjects signed an informed consent. Punch biopsies of buccal mucosa were obtained under local anesthesia at designated 4-hour intervals (0800, 1200, 1600, 2000, 2400, 0400) from six healthy male subjects (age 26 to 45, mean 34 years). All volunteers had a normal sleeping pattern and were active during the day. All of the biopsies in all six subjects were done over a single 24-hour period. The subjects slept during the night of the procedure except for the 30 minutes required to perform the biopsy at midnight and 0400. Subjects ate at their normal meal times. Using a syringe, 0.5 ml of 4% prilocaine without epinephrine was injected into the biopsy site. A 5-mm dermatological punch was used to obtain a core biopsy composed of epithelium and the underlying connective tissues. Biopsies were taken from the occlusal line of the buccal mucosa alternating between the right and the left side and avoiding areas of previous biopsy. Once biopsied, the core of tissue was immersed immediately in 10% neutral-buffered formalin. Following a uniform fixation period of 12 hours, the specimens were processed to paraffin. Five-μm thick tissue sections were cut onto glass slides, coated with 2% aminopropylethylsilane (Sigma, St. Louis, MO) and stored at 4°C. All sections were immunostained within 24 hours of microtome cutting. A hematoxylin and eosin-stained tissue section was reviewed in each case for adequacy of tissue and preservation of tissue morphology.

Immunohistochemistry

Standard immunohistochemistry[21] coupled with pressurized heat antigen retrieval[22] was used to identify epithelial cells showing nuclear staining for the proteins cyclins-A, -E, -B1, Ki-67, and p53 (see Figure 1). Briefly, the tissue sections were dewaxed in xylene and rehydrated in graded ethanols. Endogenous peroxidase activity was blocked by quenching the sections in 0.3% methanolic peroxide. For antigen retrieval, the sections were heated in a pressure cooker to 130°C for 2 minutes and then quenched in water.[22] The specificities of the monoclonal antibodies used are listed in Table 1. The bound complexes were detected by the application of preformed avidin-biotin complexes, and the final product was detected by diaminobenzidine precipitation according to manufacturers specifications (Vector Laboratories, Burlington, Canada). The sections were lightly counterstained with hematoxylin.

A computer image analysis system (IBAS System, Kontron Electronik, Dusseldorf, Germany) was used to accurately quantify the number of positive cells in the basal and parabasal layers of the epithelium in each sample. For each section, the length of basement membrane was measured in mm at a magnification of $\times$20 . Following this, at a magnification of $\times$200, all cells showing nuclear staining were identified in the epithelium and counted for each region. This was repeated until the entire length of the epithelium was analyzed. The number of positive cells was then calculated for each specimen and expressed as the number of positive cells per mm of basement membrane. Because dividing cells will show both nuclear and cytoplasmic staining for cyclin-B1 protein, only nuclear expression was assessed for this protein.

Data Analysis

The single cosinor method[23–25] was used to analyze for circadian rhythm individually and as a group, using both original units and normalized data (percent of individual means). This inferential method involves fitting a cosine curve of a predefined period (24 hours in this case) by the method of least squares. The rhythm characteristics and their dispersions' standard error (SE) and 95% confidence interval (CI)) estimated by this method include the mesor (middle value of the fitted cosine representing rhythm adjusted mean), the amplitude (half the difference between the minimum and maximum of the fitted cosine function), and the high point or acrophase (time of peak value in the fitted cosine function). A *P* value for rejection of the zero-amplitude (no rhythm) assumption is determined for each data series, indicating whether or not the cosine model accounts for a significantly greater proportion of the variability in the time series when compared with the total variability around a flat line (the overall

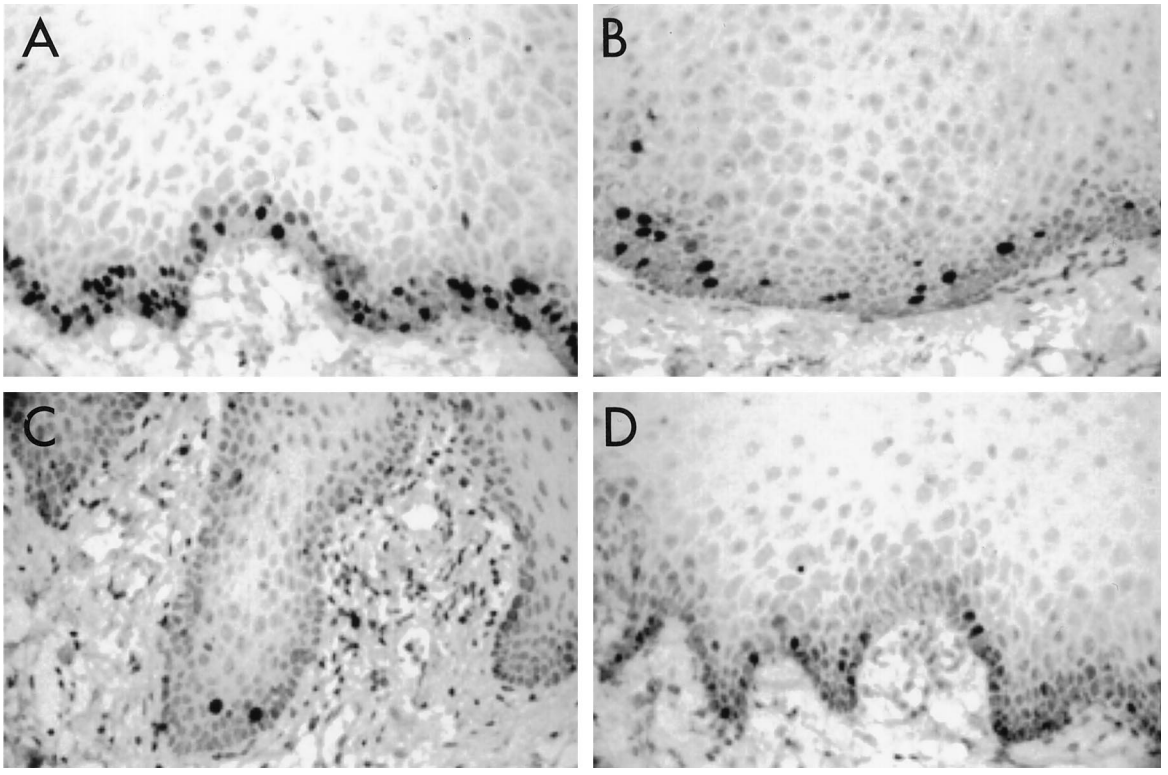

Figure 1. Immunohistochemical detection of cell-cycle proteins in the basal and parabasal layers of normal human oral epithelium. A: Ki-67; B: cyclin A; C: cyclin B1; D: p53.

mean). Rhythm detection is considered statistically significant with a P value of ≤ 0.05.

The range of change (ROC) within 24 hours was computed for each variable and subject by subtracting the lowest value from the highest value. This difference was also expressed as percentage of the lowest value. Paired *t*-tests were used to test for differences between lowest and highest value for each variable. Because of large interindividual differences in mean values between variables, individual data for each variable were normalized by re-expressing each value as percentage of its series mean before statistical analyses of grouped data. The six sampling time normalized means were analyzed for time-effect by one way analysis of variance (ANOVA).

Results

The data for individual subjects and the group analysis are summarized in Tables 2 and 3, respectively. The high point (maximum number of positive cells per millimeter of basement membrane) in a 24-hour period for each variable was calculated using cosinor analysis for each subject (Table 2, columns 2 through 7) and for the group as a whole (Table 3, columns 7 through 9). Several period lengths were tested, and the 24-hour period gave the best fit to the data. In the following discussion, count per millimeter basement membrane refers to the number of cells per length of basement membrane showing nuclear staining for the specified protein. Figure 2 shows the cosinor analysis graphs for p53 using both the original data and the normalized data. Figure 3 shows the cosinor analysis graphs for cyclins-A, -E, -B1, and Ki-67 using the normalized data. Table 4 shows the raw data for cyclin A and p53.

The group high point for p53 nuclear staining was at 10:56 hours (95% CI: 08:12 hours to 13:44 hours, $P = 0.016$ for rhythm). The mean count (of p53 positive cells) per millimeter was 17.5 with a mean variability from the lowest to the highest count of 12.68 ($P < 0.001$). The mean range of change from lowest to highest count was 131% with individual ranges of change from 73 to

Table 1. Antibody Specificities and Concentrations

Antibody	Source	Clone	Type	Dilution	Cell-cycle specificity
Ki-67	Novocastra*	MM1	monoclonal	1:1000	Proliferating cells
Cyclin E	Santa Cruz†	HE12	monoclonal	1:5000	Peaks at G1/S
Cyclin A	Novocastra	6E6	monoclonal	1:1000	Peaks in G2
Cyclin B1	Novocastra	GNS-1	monoclonal	1:500	Peaks in M
p53	Novocastra	DO7	monoclonal	1:500	Wild-type, and mutant at p53 protein, late G1

* Novocastra Laboratory, Newcastle, UK.
† Santa Cruz Laboratory, Santa Cruz, CA.

Table 2. Timing of Calculated High Points of Nuclear Expression of Cell-Cycle-Associated Proteins for Each Subject over 24 Hours

Variable	Subject 1 (hours)	Subject 2 (hours)	Subject 3 (hours)	Subject 4 (hours)	Subject 5 (hours)	Subject 6 (hours)
p53	10:13	11:45	14:51	07:20	06:41	14:20
Cyclin E	15:12	14:00	15:55	13:50	15:00	15:17
Cyclin A	15:08	14:50	16:51	13:13	19:14	17:15
Cyclin B1	20:41	19:01	22:38	23:44	22:59	17:13
Ki-67	01:54	22:40	03:13	06:40	01:08	08:59

All Subjects had biopsies every 4 hours over 24 hours during the same 24-hour period.

262%. Wild-type p53 protein is detected in the nucleus in late G1.[26–28]

The group high point for cyclin E nuclear staining was at 14:59 hours (95% CI: 14:08 hours to 15:48 hours, $P < 0.001$ for rhythm). The mean count per millimeter was 0.53 with a mean variability from the lowest to the highest count of 0.67 ($P < 0.002$, mean range of change 264%; individual ranges of change 138 to 405%). Cyclin E is detected in the nucleus in late G1-phase and peaks at the G1/S boundary.[29]

The group high point for cyclin A nuclear staining was at 16:09 hours (95% CI: 14:08 hours to 18:00 hours, $P < 0.001$ for rhythm). The mean count per millimeter was 54.7 with a mean variability from the lowest to the highest

Table 3. Nuclear Expression of Five Cell-Cycle-Associated Proteins in Oral Epithelium: Combined Raw Data and Normalized Data for All Six Subjects

	Raw data for the group: 24-hour mean and range of change					Normalized data for the group: Cosinor analysis and ANOVA			
Variable	24-hour mean ± SE count/mm	Mean ROC*	Paired *t*-test† for ROC	Mean ROC* %	Individual ROC* %	Group high point (hr:min)	95% CI for high point (hours)	*P* value for rhythm by Cosinor	*P* value for time effect by ANOVA‡
p53	17.5 ± 1.0	12.68	<0.001	131	73–262	10:56 hours	08:12–13:44	0.016	0.064
Cyclin E	0.53 ± 0.05	0.67	0.002	264	138–405	14:59 hours	14:08–15:48	<0.001	0.001
Cyclin A	54.7 ± 4.8	41.36	0.002	130	63–207	16:09 hours	14:08–18:00	<0.001	0.001
Cyclin B1	1.20 ± 0.14	1.45	0.014	362	95–1175	21:13 hours	18:36–23:56	0.016	0.058
Ki-67	73.1 ± 5.8	47.72	0.003	108	32–188	02:50 hours	00:08–05:28	0.012	0.012

* ROC, range of change from lowest to highest value.
† *t*-test comparing peak *versus* trough.
‡ One-way analysis of variance.

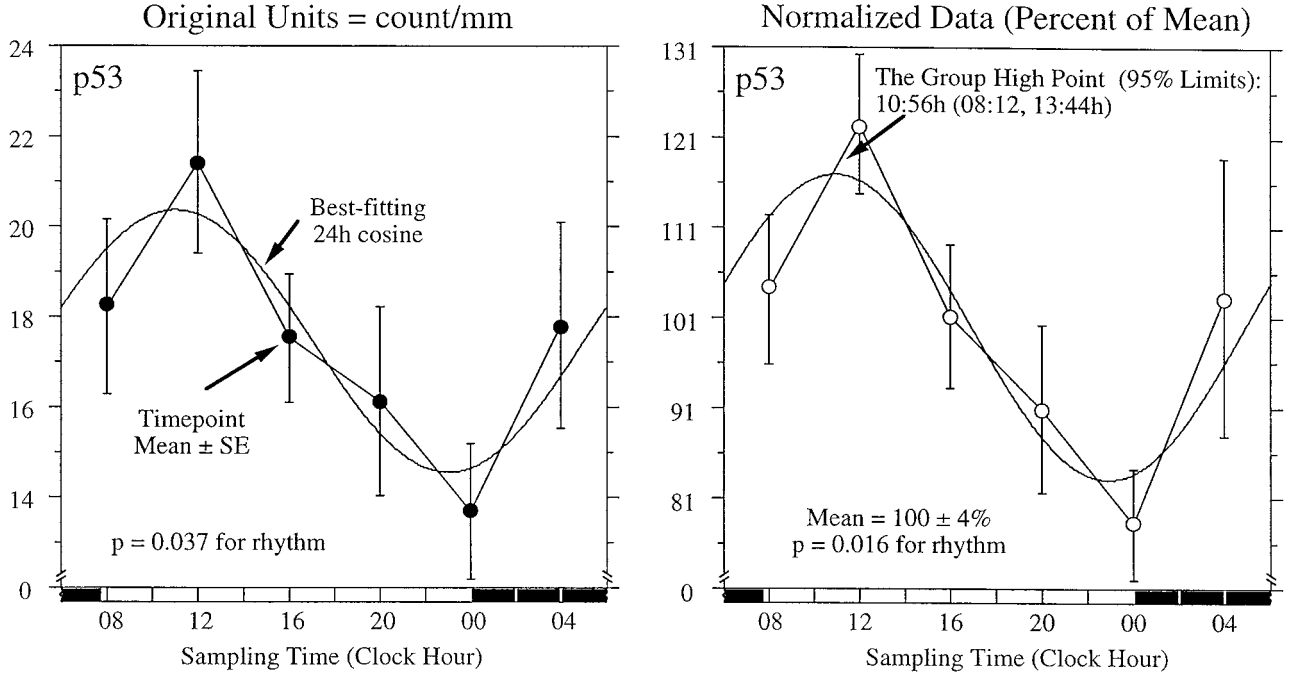

Figure 2. Circadian variation in oral mucosa p53 in six subjects, showing both original data (left panel) and normalized data (right panel). Time point means (original value and normalized value) and SE of protein expression is depicted along the 24-hour time scale. The best fitting cosine curve is shown in both panels. The time for the high point of expression, overall mean, and *P* value for rhythm detection is shown. h, hours.

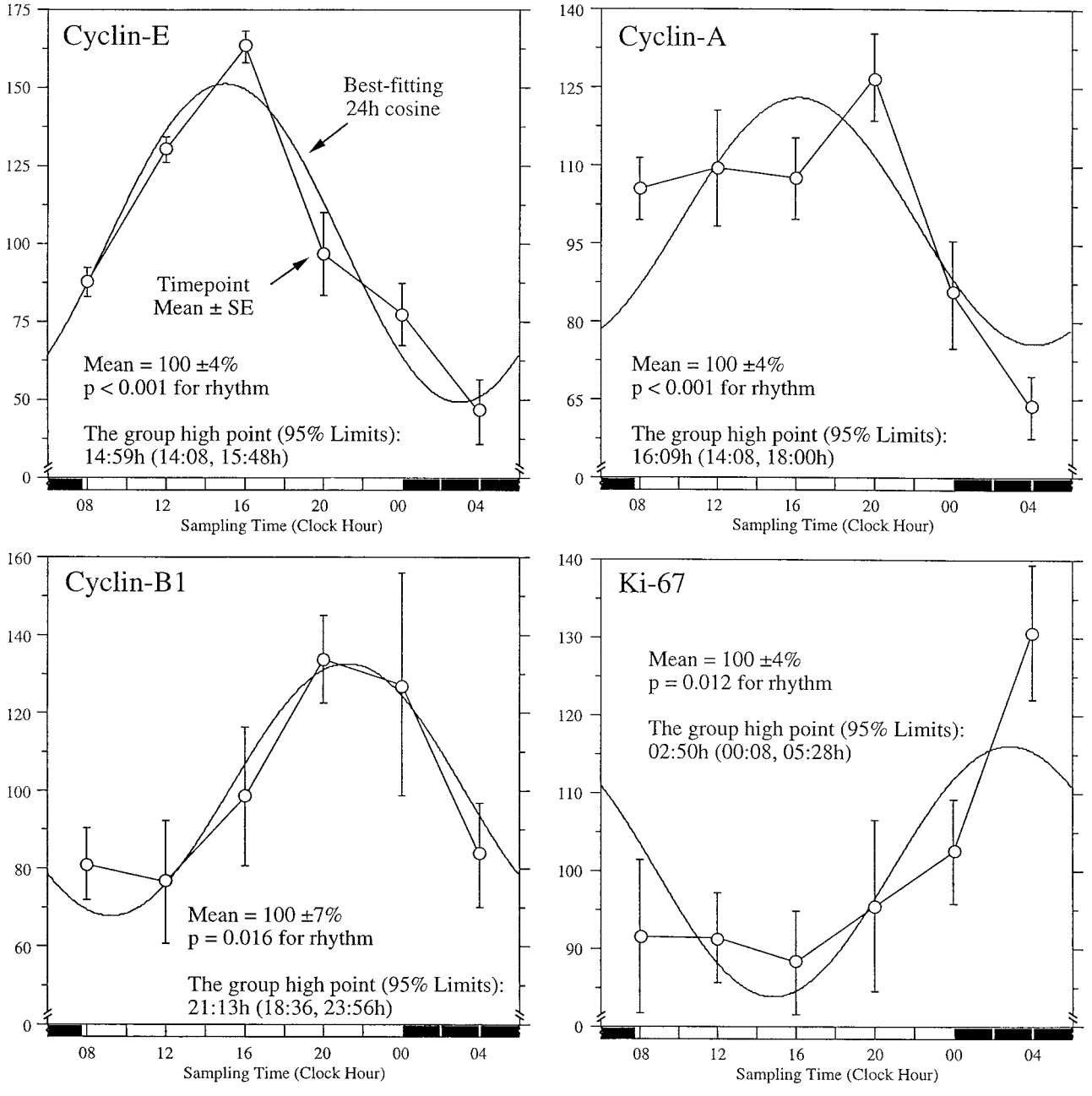

Figure 3. Circadian variation in oral mucosa cyclin-E, cyclin-A, cyclin-B1, as well as Ki-67 in six subjects, using normalized data. Time point means (expressed as percentage of mean) and SE of protein expression is depicted along the 24-hour time scale. The best fitting cosine curve is shown. The time for the high point of expression, overall mean, and *P* value for rhythm detection is shown. h, hours.

count of 41.36 ($P = 0.002$, mean range of change 130%; individual ranges of change 63 to 207%). Cyclin A appears in the nucleus in S phase and peaks in early G2 phase.[30,31]

The group high point for cyclin B1 nuclear staining was at 21:13 hours (95% CI: 18:36 hours to 23:56 hours, $P = 0.016$ for rhythm). The mean count per millimeter was 1.2 with a mean variability from the lowest to the highest count of 1.45 ($P = 0.014$, mean range of change 362%; individual ranges of change 95 to 1175%). Nuclear staining for cyclin B1 is detected in M phase.[32,33]

The group high point for Ki-67 nuclear staining was at 02:50 hours (95% CI: 00:08 hours to 05:28 hours, $P = 0.012$ for rhythm). The mean count per millimter was 73.1 with a mean variability from the lowest to the highest count of 47.7 ($P = 0.003$, mean range of change 108%; individual ranges of change 32 to 188%). Ki-67 is a poorly characterized, nuclear, nonhistone protein that is expressed in proliferating cells.[34,35]

For the group as a whole, the normal physiological order of peak protein expression over time is evident from these data. Figure 4 depicts this, showing the high point

Table 4. Raw Data for Cyclin A and p53 Expression in Oral Mucosa

Subject	Time of day when biopsy done	Number of nuclei positive for Cyclin A per mm BM	Number of nuclei positive for p53 per mm BM
1	0805	46.55	19.41
	1152	39.61	21.65
	1550	55.40	15.27
	1954	45.68	16.63
	2350	34.90	12.02
	0348	34.09	17.61
2	0800	47.98	23.68
	1156	73.31	29.50
	1601	42.15	17.64
	2005	64.40	21.45
	2355	49.77	20.33
	0400	41.87	16.25
3	0803	27.72	14.71
	1201	64.62	20.59
	1552	46.40	17.87
	1950	59.22	15.83
	2357	45.93	14.79
	0356	22.39	7.84
4	0750	72.62	15.47
	1158	65.93	17.08
	1547	58.80	14.04
	1948	69.89	6.49
	0005	41.37	10.80
	0350	41.34	23.52
5	0755	51.95	23.58
	1154	43.20	15.80
	1559	55.27	16.34
	2000	89.36	17.90
	2359	56.84	13.66
	0354	29.09	22.23
6	0752	81.41	12.45
	1148	66.33	23.82
	1558	97.18	23.94
	1958	87.16	18.42
	0002	83.67	10.46
	0355	35.56	19.30

BM, Basement membrane.

and 95% confidence interval for each variable over 24 hours. For each individual subject the normal physiological order of peak protein expression over time is also largely maintained (Table 2). Exceptions (shown underlined) to the normal physiological progression include cyclin A and cyclin B1 in subject 6 in which the high points are close in time. The high points for cyclin E and cyclin A in subjects 1 and 4 are also close in time. The counts per millimeter for both cyclin E and cyclin B1 were low, probably because of their short half-lives. The low counts make determination of the peak times for cyclin E and cyclin B1 less reliable than for the other proteins. As expected, there is some interindividual variation with regard to the timing of peak protein expression for each variable.

Discussion

Our study is the first to examine the nuclear expression of cell-cycle-associated proteins over a 24-hour period in

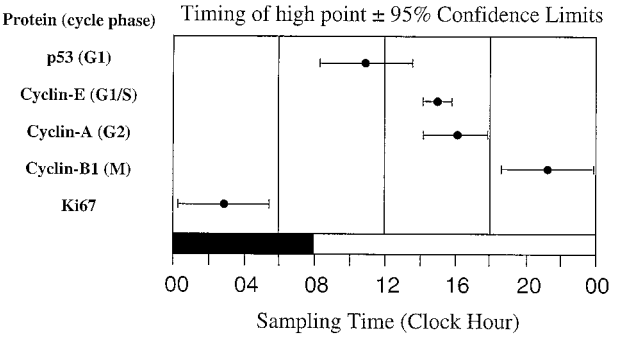

Figure 4. The high point for the best-fitting cosine for p53, cyclin-E, cyclin-A, cyclin-B1, and Ki-67 is depicted along the 24-hour time scale. The 95% confidence limit for each variable is shown. The cell-cycle phase that each protein is a marker for is shown in parenthesis. The normal physiological progression from one cell-cycle phase to the next is evident in this figure.

unperturbed, anatomically intact, human epithelium. The number of nuclei stained for each of the different cell-cycle proteins varies significantly with the time of day. Furthermore, the circadian pattern of staining for each cell-cycle marker is unique. The time of day for maximum p53 staining (late G1) was in the morning (11:00 hours). This was followed 4 hours later (15:00 hours) by maximum cyclin E staining (G1/S marker), and 5 hours later (16:00 hours) by maximum cyclin-A staining (G2 marker), whereas the maximum staining for cyclin B1 (M marker) was 10 hours later at 21:00 hours in the evening. This data gives further strong support to the concept that circadian time coordinates cell-cycle dynamics. The cycling cell population in normal tissues, such as the oral epithelium, appears as a group to go through the cell cycle in a synchronous fashion with a greater proportion of cycling cells entering the same phase of the cell cycle at around the same time of day. The appreciation that the molecular events associated with cell proliferation in normal human tissue are synchronized in time may lead to a better understanding of cell-cycle progression in health and disease. That statistically significant results were obtained for all parameters in a group of only six subjects emphasizes the strength of this phenomenon. As is to be expected, there were some interindividual variations in the timing of cell-cycle events as the circadian system is coordinated to a large extent by the time of sleep onset.[36] All subjects had normal but not identical sleeping habits.

The earliest studies looking at the rhythmic nature of cell proliferation in rodent gut were based on mitotic counts and were very time consuming.[37] With the advent of radioisotopes, it became possible to study cell proliferation by measuring the uptake of tritiated thymidine, which reflects the S phase of the cell cycle. More recently flow cytometry has been applied to study this phenomenon in the bone marrow.[17] This technique does not work well for gut mucosa because it requires a single cell suspension.

Scheving et al[38] have studied the mitotic index and the uptake of tritiated thymidine in rodent gut mucosa extensively. In studies on different strains of mice, a significant circadian variation was found for these parameters in the epithelium of tongue, esophagus, stomach, jejunum, and rectum.[12,13] The phasing of the rhythms in the different

regions of the gut were remarkably similar with the peak in DNA synthesis occurring around the time of transition from dark to light in the animal cage (late activity). There was approxiamtely a 1 to 2 hour delay in the high point of DNA synthesis in the rectum compared with that of esophagus. With fasting, there was a decrease in the amplitude of the rhythms, but the phasing persisted for up to 72 hours.[39,40]

Two studies have looked at the proliferation in human rectal mucosa biopsies over 24 hours.[14,15] In the first study rectal biopsies were obtained every 2 or 3 hours for 24 hours from 16 volunteers under fasting and fed conditions and incorporation of tritiated thymidine (by scintillation) measured in each specimen. Both fasted and fed subjects showed significant circadian rhythms in thymidine incorporation that peaked at 07:00 hours in the morning (early activity).[14] Fasting lowered the thymidine incorporation but did not change the rhythm phase. Whereas the impact of fasting is in agreement with the rodent data above, the timing of S phase is different (late activity *versus* early activity). In this study the cellular architecture was not preserved, and therefore the site within the rectal crypt where proliferation was occurring could not be determined. Fibroblasts, histiocytes, and lymphocytes from the tunica propria would also have been included in the study sample.

The second study was designed to investigate circadian proliferation within the rectal crypt epithelium and to locate the sites of fluctuation along the longitudinal axis of the gland.[15] In this study, rectal biopsies were taken every 4 hours over 24 hours from 23 normally fed subjects. Using tritiated thymidine autoradiography, a circadian rhythm was found in the labeling index with a peak at 01:28 in the morning (late activity, consistent with rodent data). The circadian rhythm was almost entirely accounted for by fluctuations in the labeling index in the compartment of the crypts normally associated with cell replication.[41] The base of the crypt as well as the upper 40%, which contains mainly differentiated cells, showed no circadian variation in labeling index. The fact that the results from these two studies are not in agreement with regard to the timing of S-phase, may have to do with the different techniques used for thymidine labeling and the limitations of this method in general.[42] The only human study examining the labeling index in human oral mucosa has reported a significant time of day variation.[16] In this study, three subjects had biopsies at six times on three different days, and eight subjects provided only two samples at two different times of day. For the three subjects studied at six times, there was a peak at 22:00 hours in the evening (late activity). A cosinor analysis was not used in this study. There are no rodent data looking at oral mucosa proliferation. Our data show a greater proportion of cells in S phase during the middle of the activity phase.

The method used in our study has several advantages. It avoids the potential confounding effects that might be introduced by subjecting samples to *in vitro* labeling or by using *in vivo* labeling agents whose clearance an/or metabolism might also contribute to circadian timed effects.[42] Several studies have quantitated either S phase or M phase fraction in a given tissue around the clock, but no previous study has looked at both processes at the same time as well as the G1 and G2 phase. Finally, the ability to delay the majority of the sample processing to later improves the feasibility of performing a study like this.

Endogenous biological rhythms have been demonstrated at all biological levels, from yeast and nucleated unicells to man, and at all levels of biological organization.[36] Of the established rhythm periods, the circadian rhythm (24-hour period) has been most thoroughly investigated.[43,44] The suprachiasmatic nucleus (SCN) coordinates the circadian rhythm, and through its connections to the retina allows synchronization to changes in the sleep activity routine.[45] Pineal melatonin synthesis is driven by the circadian neural activity of the SCN, being low during the day but high at night.[46] The genetic control of the circadian clock is the subject of intense research and rapid progress at the present time.[47,48] A recent study has show that serum shock induces circadian gene expression in fibroblasts and hepatoma cells in culture and that these cells express the same clock genes that are expressed in the SCN.[49] These data suggest that each organ may have the ability to control its circadian rhythm by an endogenous clock mechanism but requiring some central input form the suprachiasmatic nucleus to maintain rhythmicity. Our findings raise the question how the circadian variation in cell-cycle progression at the tissue level is controlled by the human circadian clock. Edmunds and colleagues have studied this question in *Euglena gracilis* and have suggested that bimodal circadian changes of cAMP and cGMP levels may be essential for the control of the cell cycle by the circadian clock.[50,51]

Ki-67

Analysis of the expression of Ki-67 is used widely to determine the proliferative index of the cells in normal and tumor specimens.[52] Our study, using an antibody to an epitope of the Ki-67 protein, has found peak nuclear protein expression in the early morning (03:00 hours), 6 hours after the peak for cyclin-B1 (M marker) and 8 hours before the peak for p53 (late G1 marker). Scott et al[53] have shown that a large number of G1 cells are stained *in vivo* by Ki-67.[53] Cells in G0 are negative for Ki-67 but become positive during the transition from G0 to G1 and remain positive in S, G2, and M. It may be postulated that the peak for Ki-67 identified in our study reflects a population of epithelial cells that are re-entering the cell cycle from a nonproliferative state during G0.[54] Cells undergoing differentiation and apoptosis are also negative for Ki-67. The low point for Ki-67 might therefore represent a time of day when more cells are undergoing differentiation and apoptosis.

p53

The role of p53 protein in normal cell-cycle progression is not well understood. This protein has been shown to act as a transcription factor important in the control of G1

cell-cycle arrest and apoptosis in the setting of DNA damage.[55] The finding of a circadian variation in the nuclear expression of p53 protein at a time corresponding to late G1, suggests that p53 may also play a role in normal cell-cycle progression. The observed rhythmic expression of p53 protein may have to do with a surveillance function regarding genomic integrity before entry into S phase. Alternatively this observation may reflect a role for p53 in differentiation.[56,57] Terminal differentiation, associated with loss of proliferative activity, is often accompanied by growth arrest in G1 and sometimes subsequent apoptosis. The high cell turnover in the oral epithelium would be consistent with this possibility. Finally, it may be hypothesized that p53-dependent apoptosis may be rhythmic in concert with the rhythmic expression of p53.

Clinical Implications

The ability to time certain events in the cell cycle based on the peak expression of the different cyclin proteins is a potentially valuable research tool. In malignant tissue, the level of expression of cyclins and their inhibitors may have important prognostic and therapeutic implications.[58,59] Our data suggest that the time of day of tissue sampling may impact on the ability to detect high or low levels of the different cell-cycle proteins.

The cell-cycle phase-dependent toxicity of several chemotherapy drugs is well established. Experimental and clinical studies have shown that the time of delivery of over 20 chemotherapy drugs impacts on normal tissue toxicity and activity.[60,61] Two prospective randomized clinical trials, in patients with metastatic colorectal cancer, have now confirmed that the timing of 5-fluorouracil-based chemotherapy significantly impacts both on its antitumor activity and the incidence of oral mucositis.[62,63] In the most recent and larger study, time modified delivery of chemotherapy improved the therapeutic index significantly with a better objective response rate (51 *versus* 29%, $P = 0.003$) and a fivefold reduction in the rate of severe mucositis (14 *versus* 76%, $P < 0.0001$). The circadian rhythm in oral mucosa proliferation reported here may in part be responsible for this finding. This is supported by data from Sonis et al[64] showing that topical mucosal application of transforming growth factor-β3, which reduces the fraction of cycling basal epithelial cells, significantly reduces 5-fluorouracil-induced mucositis in hamsters.

Studies of synchronously dividing cells in culture have revealed greatest radiosensitivity for cells in G2 and M phases, relative radioresistance for cells in late S phase, and intermediate radiosensitivity between these two extremes.[65–67] There is experimental data to suggest that the toxicity from radiotherapy is time dependent for several normal tissues and for animal lethality.[68–73] Studies have also documented the time-dependent ability of radiation to generate apoptosis in the gut mucosa.[74–76] When mice were irradiated at different times of day a clear circadian rhythm was observed in the number of apoptotic cells in the intestinal crypts. The best treatment time for inducing apoptosis was in the morning (late activity phase) coinciding with a time when most of the target cells were in G2-M. The trough occurred with treatment in the evening (early activity phase) coinciding with a time when most of the target cells were in G1.[74] Rodent studies have also shown that the mitotic delay in response to radiation does not change the circadian variation in cell proliferation.[69] Based on the data presented here, the most radiosensitive phase of the cell cycle (G2-M) occurs in late afternoon/evening in human oral mucosa. Therefore, radiation therapy including the oral cavity may be associated with less oral mucositis when administered in the morning as compared with the late afternoon. A clinical trial is ongoing to test this hypothesis.

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