

Cellular Proliferation Potential During Aging and Caloric Restriction in Rhesus Monkeys (*Macaca mulatta*)

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Caloric restriction (CR) is the most successful method of extending both median and maximal lifespans in rodents and other short-lived species. It is not yet clear whether this method of life extension will be successful in longer-lived species, possibly including humans; however, trials in rhesus monkeys are underway. We have examined the cellular proliferative potential of cells from CR and AL (ad libitum fed) monkey skin cells using two different bioassays: colony size analysis (CSA) of dermal fibroblasts isolated and cloned directly from the skin and beta-galactosidase staining at pH 6.0 (BG-6.0) of epidermal cells in frozen sections of skin. Decreases in both proliferative markers occurred with age, but no differences were observed between CR and AL animals. Skin biopsies were obtained from AL and CR rhesus monkeys from two different aging colonies, one at the National Institute on Aging (NIA) and one at the University of Maryland-Baltimore (UMB). These biopsies were used as a source of tissue sections and cells for two biomarkers of aging assays. The CR monkeys had been maintained for 9–12 years on approximately 70% of the caloric intake of control AL animals. In the CSA studies, the fraction of small clones increased significantly and the fraction of large clones decreased significantly with increasing age in AL monkeys. The frequency of epidermal BG-6.0 staining cells increased with age in older (>22 years) AL monkeys, but most predominately in those of the UMB colony, which were somewhat heavier than the NIH AL controls. Old monkeys on CR tended to have fewer BG-6.0-positive cells relative to old AL-derived epidermis, but this effect was not significant. These results indicate that cellular proliferative potential declined with age in *Macaca mulatta*, but was not significantly altered by CR under these conditions. Although these experiments are consistent with an absence of effect of CR on monkey skin cell proliferative potential, we have found in previous experiments with mice that a longer duration of CR (as a fraction of total lifespan) was needed to demonstrate CR-related improvement in clone size in mice. Further studies on the now mid-aged monkeys will be needed as their age exceeds 20 years to conclusively rule out an effect of CR on proliferative potential of skin cells from these primates. J. Cell. Physiol. 180:123–130, 1999.

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Caloric restriction (CR) is the most successful method of lengthening the median and maximal lifespans of laboratory rodents as well as many other short-lived species (for reviews see Weindruch and Walford, 1988; Yu, 1994a,b). Studies in longer-lived nonhuman primates have been underway for over a decade to address the important question of whether CR has similar effects in species more closely related to humans (Ingram et al., 1990; Lane et al., 1992; Kemnitz et al., 1993; Hansen and Bodkin, 1993; Ortmeier et al., 1994; Bodkin et al., 1995). To date, studies in rhesus monkeys demonstrate clearly that the majority

of physiological responses seen in monkeys on CR agree with previous findings in rodents (reviewed in Lane et al., 1997a).

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TABLE 1. Design of study

Monkey colony group	Clone size			Beta gal		
	Age (yr)	N	Body weight (kg)	Age	N	Body weight (kg)
NIA						
Adult						
AL	12 $\pm$ 0.4	7	10.0 $\pm$ 0.7	11 $\pm$ 1.0	14	10.4 $\pm$ 0.7
CR	12 $\pm$ 0.4	9	7.7 $\pm$ 0.7	11 $\pm$ 1.0	14	8.2 $\pm$ 0.5
Old						
AL	28 $\pm$ 0.3	2	8.3 $\pm$ 0.9	25 $\pm$ 0.8	8	9.0 $\pm$ 0.6
CR	29 $\pm$ 2.5	2	6.6 $\pm$ 1.2	27 $\pm$ 1.0	7	7.2 $\pm$ 0.4
U of MD						
Adult						
AL	11 $\pm$ 0.8	5	18.2 $\pm$ 2.6	10 $\pm$ 1.0	2	16.8 $\pm$ 1.8
CR	—	—	—	—	—	—
Old						
AL	23 $\pm$ 2.3	6	15.8 $\pm$ 2.0	24 $\pm$ 2.0	4	15.6 $\pm$ 2.8
CR	25 $\pm$ 0.8	6	10.3 $\pm$ 0.1	26 $\pm$ 0.3	3	10.4 $\pm$ 0.1

We studied two potential biomarkers of skin cell aging in two separate rhesus monkey (*Macaca mulatta*) colonies presently undergoing long-term CR. These potential biomarkers are measures of cellular proliferative reserve. They include 1) a colony size distribution assay (CSA) analysis of dermal fibroblasts cloned directly from primary isolates of cells and 2) beta-galactosidase activity at pH 6.0 (BG-6.0) of epidermal cells in frozen sections from some of the same samples. CSA has been used by us and others to detect losses of cellular proliferative potential during aging in rodents, dogs, and humans (Smith et al., 1978; Pendergrass et al., 1995; Wolf et al., 1995; Li et al., 1996, 1997, 1998). It has also been used during in vitro aging of human cells (Martinez et al., 1978; Smith et al., 1978; Angello et al., 1987; Angello and Prothero, 1989). CSA has also been used to detect replication-preserving effects of CR in many different mouse tissues (Pendergrass et al., 1995; Reed et al., 1996; Li et al., 1997, 1998).

The fraction of both human skin epidermal and dermal cells expressing BG-6.0 has been shown previously to increase with the age of the donor (Dimri et al., 1995). This biomarker also stains nongrowing senescent cells that accumulate during in vitro aging and is one of a few biomarkers that is sensitive to aging both in vitro and in vivo (Dimri et al., 1995). The present study is the first attempt to stain skin samples for BG-6.0 from nonhuman primates.

In the present study, the fraction of large clones decreased and the proportion of small clones increased with age in dermal fibroblasts from ad libitum fed (AL) monkeys, but no significant differences between the CR and AL diet groups were detected. Similarly, we observed an age-related increase in the incidence of BG-6.0-positive epidermal cells in AL monkeys. Old CR monkeys displayed a trend toward a lower incidence of BG-6.0-positive cells than AL controls; however, this effect was not statistically significant.

MATERIALS AND METHODS

Subjects

The data were obtained from two subsets of monkeys in separate ongoing studies of CR, one at the National Institute on Aging (NIA; Ingram et al., 1990) and the other at the University of Maryland-Baltimore (UMB;

Hansen and Bodkin, 1993). Extensive details on housing and animal husbandry in these colonies have been published in previous reports (Ingram et al., 1990; Hansen and Bodkin, 1993; Bodkin et al., 1995; Hansen et al., 1995; Lane et al., 1995). All monkeys were housed individually in standard rhesus monkey caging. Monkeys in both studies were fed a pelleted diet either two (NIA) or three (UMB) times a day. The diet used by the NIA is similar to standard monkey, except that a vitamin and mineral premix is added during manufacture of the chow. Monkeys in the UMB colony received standard monkey and a daily chewable vitamin supplement. Specific information on diet formulation can be found in previous reports (Ingram et al., 1990; Hansen and Bodkin, 1993; Lane et al., 1995).

Monkeys in both colonies were assigned to either the control (AL) or the CR group. Monkeys were also divided into two age groups. When these studies were conducted, the adult group consisted of monkeys approximately 9–12 years of age and the monkeys in the old group were all over the age of 20 years. Monkeys demonstrating diabetic blood glucose levels were excluded from the study. Regular measurement of food intake has shown that the meal allotments offered to AL monkeys in both studies approximate ad libitum feeding. The UMB control monkeys were somewhat heavier than the NIH control monkeys (see Table 1). Monkeys on CR at the NIA receive 30% less than controls of a comparable age and body weight. In slight contrast, CR monkeys in the UMB study were placed on a weight stabilization/calorie titration protocol on entry into the study. Intake amounts for CR monkeys were adjusted weekly to maintain a stable adult body weight. This weight stabilization protocol has resulted in an average reduction in calorie intake of about 35% relative to the fully AL monkeys (Bodkin et al., 1995). As such, the level of CR is close to 30% in both studies. It is important to note that the previous physiological data related to effects of CR reported from these two studies are consistent between the NIA and UMB groups (Lane et al., 1997a).

Measurements

Two biomarkers related to cellular (fibroblast) aging were examined: CSA and BG-6.0. Biopsies were collected from monkeys in the two above-mentioned age

groups (adult [9–12 years] and old [>20 years]) from each study. Specific numbers of monkeys used in each experiment and their average ages are summarized in Table 1. Studies of CSA utilized skin biopsies that were harvested from a total of 35 rhesus monkeys ($n = 18$ AL, 17 CR). For BG-6.0 studies, biopsies were collected from a total of 45 animals (largely from the same animals as used for the CSA analysis), with tissue samples representing 30 AL and 15 CR monkeys. All procedures were approved by the Animal Care and Use Committees at each institution. Monkeys were fasted overnight and anesthetized with either Telazol (3–5 mg/kg, intramuscularly [IM; Ft. Dodge Animal Health, Ft. Dodge, IA]) or ketamine hydrochloride (10 mg/kg, IM) prior to preparation for biopsy collection. Additional anesthesia was provided as needed during the procedure.

A suitable region of the midback was selected and a surgical shave was performed to remove all hair from the area. The biopsy site was then cleansed with Nolvasan (Fort Dodge Laboratories, Fort Dodge, IA) and a sterile gauze containing Nolvasan left in place for 3 min. Next, the area was wiped with 70% ethanol and a sterile ethanol-soaked gauze left in place for 5 min. The area was then wiped dry with a sterile gauze. A 1×2 cm elliptical incision was made, taking care to include as little subcutaneous fat as possible. The biopsy material was cut into two equal pieces, then washed four times in phosphate-buffered saline (PBS) to remove all blood. The sample was then placed in shipping media and shipped on ice to the University of Washington for further analyses.

CSA

This method was adapted from previous studies on mouse tissues (Pendergrass et al., 1995), except that primary cloning was used rather than secondary cloning. The skin sample was obtained, rinsed with PBS, and placed in sterile DMEM shipping media (Dulbecco's modified Eagle medium with 20 mM HEPES [Gibco-BRL, Grand Island, NY] buffer and no bicarbonate). The containers were processed within 24 h of initial harvesting of the biopsy. Skin biopsies were scraped carefully with a scalpel to remove adherent fat and hair, then minced into $\sim 2\text{-mm}^2$ cubes. They were digested for 40 min at 37°C with 0.1% collagenase-dispase (Boehringer Mannheim, Indianapolis, IN) in DMEM (Gibco-BRL, Grand Island, NY) medium as above, with the addition of 20% fetal bovine serum (FBS) to prevent overdigestion. To ensure complete digestion, the digestion mixture was sealed in a plastic bag and taped to the outside of a 500-ml roller bottle on a bottle rotator apparatus (Fisher Scientific, Santa Clara, CA). The roller bottle was filled part way with ~ 250 ml of H_2O to produce pressure on the tissue pieces in the bag each revolution. Cells were rinsed through one wash with versene buffer (to inactivate the proteases). Aliquots of freshly isolated cells were filtered through a $40\text{-}\mu\text{m}$ mesh to remove any clumps (Becton Dickinson, Franklin Lakes, NJ). They were then cloned directly into Corning 150- cm^2 tissue culture flasks (Corning, New York, NY) at dilutions previously determined to provide about 50–100 clones per 100-mm dish. Twenty milliliters of DMEM media were included containing 20% FBS (cloning media) and these samples were allowed to grow untouched for 10

days. They were fixed first with 10% neutral-buffered formalin for 5 min and then with 95% ethanol and then stained with crystal violet. The number of cells per clone were enumerated by eye using a dissecting microscope. The fraction of clones that either failed to divide or that divided for one to six population doublings was recorded. Both the fraction of small senescent-like clones (less than eight cells) and the fraction of large clones (more than 32 cells) were compared from each sample. Small clones of one to eight cells per clone invariably appeared to have a large senescent cell-like morphology, as well as increased nuclear size and a more random alignment of neighboring cells. This compared to typical young clones with a small cell and nuclear size and a very parallel alignment between bipolar spindle-shaped neighboring cells. Intermediate clones of 16–32 cells were of a mixed morphology containing both large senescent-looking cells, small (younger-looking) cells, and intermediate-sized cells.

BG-6.0 stain

Biopsy pieces were frozen in liquid nitrogen and cut into $20\text{-}\mu\text{m}$ frozen sections on subbed slides and kept frozen until staining. The fraction of positive cells was determined histochemically in frozen sections using the galactose analogue X-gal, which is split by the enzyme and produces a blue insoluble dye in the cell cytoplasm of positive cells (Dimri et al., 1995). The slides were thawed and rinsed once with PBS, then fixed for 2 min at room temperature with 0.2% glutaraldehyde. The samples were then stained for BG-6.0 activity at pH 6.0 (1.8 mg/ml X-gal, 35 mM citrate, pH 6.0, 4.4 mM potassium ferrocyanide, 4.4 mM potassium ferricyanide, 133 mM NaCl, 20 mM MgCl_2) as described by Dimri et al (1995), but with 20 mM MgCl_2 added to hasten darker staining. Following staining, the slides were postfixed with 10% neutral-buffered formalin for 15 min and counterstained with eosin. Coded slides were analyzed using a blind protocol, under $\times 400$ magnification. All the positive cells in each of ten $\times 400$ fields were analyzed and recorded as the average number of positive cells per 10 fields.

Statistical methods

All statistical analyses were conducted using Statview 4.5 (Abacus Concepts, Berkeley, CA). To control for assay variability between the two colonies for CSA analyses, we calculated standardized scores (z-scores) for these data prior to conducting statistical analyses. To determine age-related changes for the various parameters, Pearson product-moment correlations were calculated to assess the relationship between chronological age and clone size distribution and BG-6.0-positive staining in AL monkeys only. We assessed the effects of CR with a 2 (age) $\times 2$ (diet) analysis of variance (ANOVA). Also, since the hypothesized effect was expected to be most evident in older groups, we compared AL and CR data in the old groups only to determine whether the age-related changes were prevented by CR. $P < 0.05$ was accepted as statistically significant.

RESULTS

Table 1 summarizes the number of animals in each age and diet group. It also provides the mean ages and

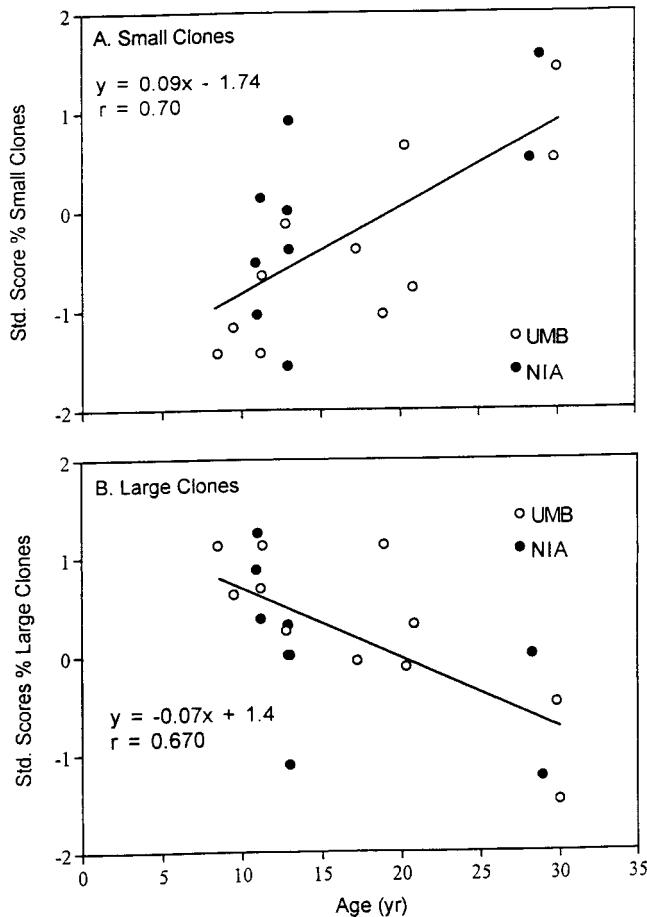


Fig. 1. Effect of age on small and large clones in AL monkeys. Each point represents the standard score (calculated based on all raw data) for the percentage of (A) small or (B) large clones. The relationship between age and the percent small and large clones was significant ($P < 0.05$).

body weights of each group. Figure 1 displays a scattergraph of clone size vs. age for all AL monkeys. Table 2 summarizes the changes in clone size distribution for each age and diet group during aging with and without chronic CR in monkeys from both colonies. Clone size measures were standardized across colonies by computing standard scores (z-scores) based on all combined raw data. Pearson product-moment correlations confirmed that small clones increased ($r = 0.70$, $P < 0.005$) and large clones decreased ($r = 0.67$, $P < 0.001$) with advancing age in AL monkeys. ANOVA showed that there were no significant effects of CR on these parameters (Table 2).

Results of BG-6.0 studies are summarized in Figure 2 and Table 3. Figure 2 shows that the number of BG-6.0-positive cells present in skin tissue sections increased with age in AL monkeys. It is noteworthy that the increase in beta-gal was not evident in all older monkeys. It occurred only after about 22 years of age and was predominately found in older monkeys from the UMB study, which were heavier than the NIH control AL monkeys. The overall staining is presented as blue epithelial cells per ten $\times 600$ fields. Approxi-

TABLE 2. Effect of CR on clone size¹

Monkey colony group	% Small ² clones	% Large ³ clones
NIA		
Adult		
AL	-0.35 ± 0.3	0.25 ± 0.3
CR	0.56 ± 0.4	-0.60 ± 0.5
Old		
AL	1.05 ± 0.5	-0.62 ± 0.6
CR	0.004 ± 0.9	0.38 ± 0.3
U of MD		
Adult		
AL	-0.96 ± 0.2	0.76 ± 0.1
CR	(none)	(none)
Old		
AL	0.69 ± 0.4	-0.12 ± 0.4
CR	-0.04 ± 0.2	0.17 ± 0.3
Combined		
Old		
AL	0.31 ± 0.3	-0.24 ± 0.3
CR	-0.03 ± 0.2	0.22 ± 0.2

¹Number of subjects tested listed in Table 1.

²Standard scores based on all small clone data from both colonies.

³Standard scores based on all large clone data from both colonies.

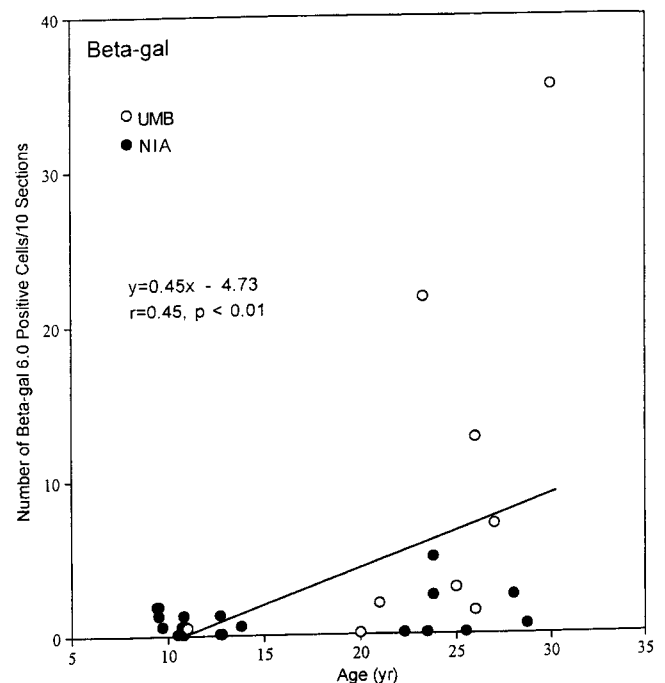


Fig. 2. BG-6.0-positive cells increased with age. Each point represents the average number of beta-gal-positive cells observed in 10 microscope sections from AL monkeys. The relationship between age and the average number of BG-6.0-positive cells was significant ($P < 0.05$).

mately 120 epidermal cells were present in each field. The staining ranged from less than 0.1% in for skin from most of the young monkeys to about 3% in the most heavily stained skin of older monkeys. Pearson product-moment correlations confirmed the significant positive correlation between age and the average number of BG-6.0-positive cells ($r = 0.45$, $P < 0.01$). Data presented in Table 3 show that monkeys on CR had less BG-6.0-positive cells compared to ALs. Although consistent among all groups and in both colonies, this

TABLE 3. Effect of CR beta-gal staining¹

Age group	NIA	UMB	Combined
Group A			
Con	0.8 ± 0.2	1.5 ± 0.1	0.9 ± 0.2
CR	—	—	—
Group O			
Con	1.7 ± 1.7	10.4 ± 6.1	6.1 ± 2
CR	1.0 ± 0.4	7.4 ± 3.0	2.8 ± 1

¹Average number of beta-gal positive cells determined in 10 microscope fields. Numbers represent the mean ± SEM. Number of subjects tested listed in Table 1.

effect was not statistically significant. For BG-6.0 studies, only data from epidermal cells were analyzed because of superior staining in this compartment. The dermal fibroblasts were not quite as distinctively stained as in human skin (data not shown) and frequently could not be separated from sectioned fragments of gland components, which stained randomly with age. Comparisons of BG-6.0 staining on cultures of monkey and human fibroblasts grown in vitro were also consistent, with less BG-6.0 activity in the monkey fibroblasts (data not shown). Figure 3 compares the BG-6.0 staining of a typical young AL monkey and that of one of the highly stained older monkeys from the UMB group. Figure 3A shows one isolated stained cell in the young epidermis typical for the young monkeys from both groups and some gland cell staining. This glandular staining is not age specific and was not analyzed (Dimri et al., 1995). In Figure 3B, a highly stained region from a sample of old AL monkey skin (from the UMB group) is shown. Several basal epidermal cells are heavily stained and some of the epidermal cells in the first few layers above the basal level are lightly stained. Several probable fibroblasts in the dermal region are also stained. As noted above, it was not always possible to separate staining of dermal fibroblasts from fragments of gland epithelium in the monkey skin, which does not increase with age, so the fibroblast staining was not analyzed.

DISCUSSION

We have confirmed that the cellular replicative potential of AL rhesus monkeys from two different colonies decreases with the age of the donor. Similar results were obtained by both CSA and the age-related appearance of the enzyme BG-6.0. However, at the time of assay in this ongoing CR study in both colonies, we found no significant differences between CR and AL diet monkeys using either of our measures of proliferative potential.

We note that most of the increase in BG-6.0 staining occurred in the three old AL monkeys from the UMB colony. These monkeys (both AL and CR) were slightly heavier than their respective groups from the NIH colony. Although we cannot rule out genetic or other environmental reasons, the heavier weights of the AL monkeys from the UMB were possibly the result of a more appetizing diet (Hansen and Bodkin, 1993). If so, the NIH controls may have been somewhat restricted relative to the UMB controls. The results of the BF-6.0 stain would be consistent with this possibility. This represents the only evidence we observed of a diet effect on proliferative potential markers. In comparing

the clone size data from the two colonies, two different batches of FBS were used to grow the clones from the NIH and UMB, respectively. The superior nature of the FBS used for the UMB study accounts for the better overall cloning of the cells from the UMB colony rather than any intrinsic cellular differences. The BG-6.0 staining of the monkey skin epithelial cells was mostly in the basal cell compartment or in early differentiating cells (Fig. 3). The fraction of cells stained ranged from <0.1% to ~3% and was usually scattered with a few foci of stained cells in the old UMB monkeys. This pattern has also been reported for human epithelial skin staining (Dimri et al., 1995).

The CSA findings on AL monkeys are consistent with previous age-related studies on cells from AL humans and AL dogs (Smith et al., 1978; Li et al., 1996). However, previous studies comparing CR vs. AL mice have demonstrated a positive effect of chronic CR on the CSA assay using cells from many different tissues from two different strains of mice (Pendergrass et al., 1995; Li et al., 1997). These same CR mice also demonstrated improved in vivo measures of cellular replicative potential relative to AL mice (Wolf et al., 1995; Li et al., 1997, 1998). We note that a single negative study in rats found that CR did not prevent age-related losses of cellular replicative potential (Pignolo et al., 1992). Our results are similarly negative to those reported in the rat study. It is possible that in monkeys and rats, CR does not increase the cellular proliferative potential. However, the mass culture population doublings (MPD) method used in that study is more subject to interference by spontaneous transformation events that occur in rodent cells, as well as overgrowth by the most proliferative subpopulation of cells, which may account for the nonsignificant findings (Pendergrass et al., 1995).

Although our data indicate that CR does not increase cellular proliferative potential in rhesus monkeys, we would like to discuss some potential obfuscating conditions that need to be addressed in future studies before a firm negative conclusion can be reached. Other important experimental factors that might obscure an effect of CR on monkey cell replicative potential include 1) the shorter relative fraction of total lifespan that the monkeys were on the dietary regimen compared to mice; 2) the old monkeys in this study were predominantly started on CR during midlife, whereas the mice in previous studies were all started on CR by 4 months of age and remained on it for at least 30 months; 3) the level of CR in the monkeys was approximately 70% of AL compared to 60% of AL for the mice. In mice, the beneficial effects of the CR regimen did not become evident in skin cells until mice placed on CR as young adults (4 months old) had been on that diet regimen for 60% of their average lifespan (Pendergrass et al., 1995). Monkeys in the present study were started at various ages and have been on CR between 38% and 50% of the species mean lifespan of about 24 years (Wood, 1991). The monkeys in the old groups were placed on CR long after they had reached sexual maturity. Thus, incipient age-related changes might have already been present at the time the CR diet was begun. If so, our data would suggest that CR cannot reverse such preexistent changes. It is possible that the significant positive effects of CR will eventually be

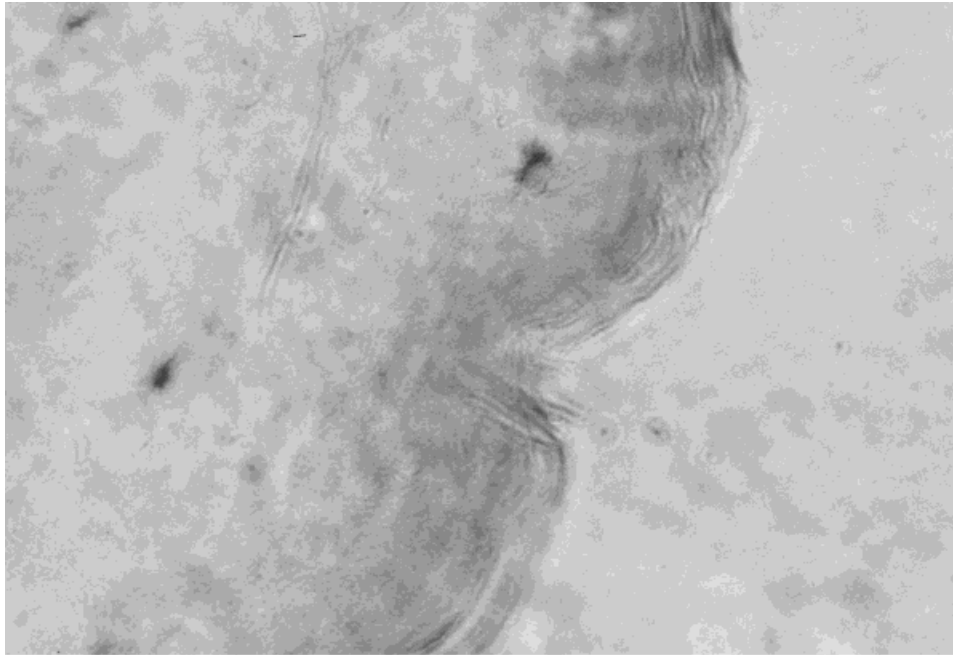
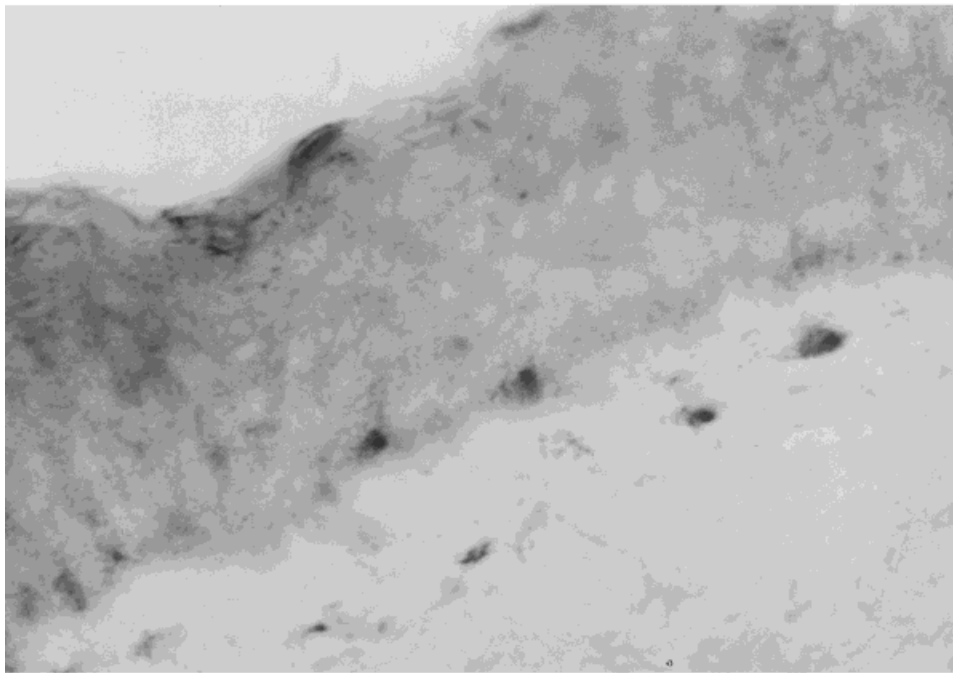
**A****B**

Fig. 3. Sample photomicrographs of BG-6.0 staining of both dermal and epidermal cells in skin sections from (A) young AL monkey skin (from the NIH group) and (B) a sample of highly stained skin from an old AL monkey from the UMB group. The slight darkening of the epidermal compartment is the result of light eosin staining to visualize this region. Rare individually BG-6.0 stained cells (A) are typical of all young monkeys from both groups and most of the older monkeys from the NIH group. Only the basal and sub-basal epidermal cells were scored as discussed in Results. Panel B demonstrates the much heavier staining seen in some focal areas from the old UMB monkeys. The dark stained area at the surface of the epithelium (B) is an artifact.

observed in monkeys started on CR at an earlier age using these same biomarkers. This could be as early as 5–10 years in the future when the monkeys started on CR as juveniles or young adults will have been on CR for greater than 60% of their mean lifespans. Finally, our findings might relate to the degree of CR. Whereas, the mice were restricted to 60% intake of AL controls, the monkeys in both studies were restricted to approx-

imately 70% of AL intake (Ingram et al., 1990; Hansen and Bodkin, 1993; Bodkin et al., 1995; Lane et al., 1995). This could further reduce the effects of CR and/or extend the length of treatment needed to induce positive changes.

The present study on BG-6.0 is the first to demonstrate the potential utility of both CSA and BG-6.0 as biomarkers of aging in nonhuman primates. The ap-

parent exponential increase in BG-6.0 positivity was seen only in UMB (AL) monkeys over 22 years of age (Table 3) and was not evident in the NIH monkeys. Despite this variable response, our findings agree generally with the increased BG-6.0 staining reported in human skin cells (Dimri et al., 1995). Monkeys on CR tended to have fewer BG-6.0-positive (senescent-like) cells, however this trend was not significant (Table 3). Overall, epidermal BG-6.0 measurement is a relatively simple procedure compared to either the CSA or MPD methods. It does not depend on tissue culture, being suitable for frozen sections. These practical advantages make it a potentially useful biomarker for exploring primate proliferative changes (Dimri et al., 1995). No studies have been published comparing the BG-6.0 staining of CR and AL rodent skin because very little BG-6.0 activity is present in rodent skin (Dimri et al., 1995; our own unpublished findings).

A number of positive effects on health and other potential biomarkers of aging have already been noted in CR rhesus monkeys in both colonies. These include 1) prevention of late-onset (type II) diabetes (Hansen and Bodkin, 1993); 2) improvement in insulin sensitivity (Bodkin et al., 1995); 3) reduction of triglycerides and increased HDL_{2b}, a high-density lipoprotein (HDL) subfraction associated with protection from cardiovascular disease (Verdery et al., 1997); 4) a slowing of the age-related decline in serum dehydroepiandrosterone sulfate (DHEAS) levels (Lane et al., 1997b); and 5) reduction in body temperature (Lane et al., 1996). Similar positive physiological effects of CR have been reported for rodents (Weindruch and Walford, 1988; French, 1992; Yu, 1994a,b; Weindruch, 1996).

Wound closure studies have been carried out in both mice and monkeys comparing CR and AL animals. The rate of wound closure, a process dependent on cellular replicative potential, was improved in old CR mice relative to old AL controls when the old CR animals were allowed to resume eating ad libitum for 2–4 weeks prior to wounding (Reed et al., 1996, 1998). The in vitro proliferative potential of cells from the unwounded skin of the old CR mice also demonstrated increased replicative potential relative to the old CR category without refeeding. This added reserve could be expressed in vivo apparently only after sufficient food was given for 2–4 weeks (Wolf et al., 1995; Reed et al., 1996). During in vitro CSA studies, optimum nutrition is always provided by the FBS and DMEM. The cells from CR animals were superior even without refeeding (Reed et al., 1996). A similar study on the rate of wound healing in rhesus monkeys demonstrated an age-related delay in the rate of healing but a nonsignificant trend toward improvement in the healing rate in animals maintained on CR (Roth et al., 1997). The refeeding (CR to AL) protocol was omitted in the monkey wound healing studies in order to maintain the required status of the ongoing diet study. Thus, the monkey and mouse wounding studies produced similar age-related findings within the context of the conditions tested. In summary, the present study provides evidence that the cellular proliferative potential and BG-6.0 of skin epidermal cell staining decrease during aging of rhesus monkeys, but no significant effect of CR on these parameters was detected. However, for the reasons discussed above, we cannot conclude that evi-

dence of CR will not be found following a longer duration of treatment with chronic CR for at least 60% of the lifespans of these nonhuman primates.

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