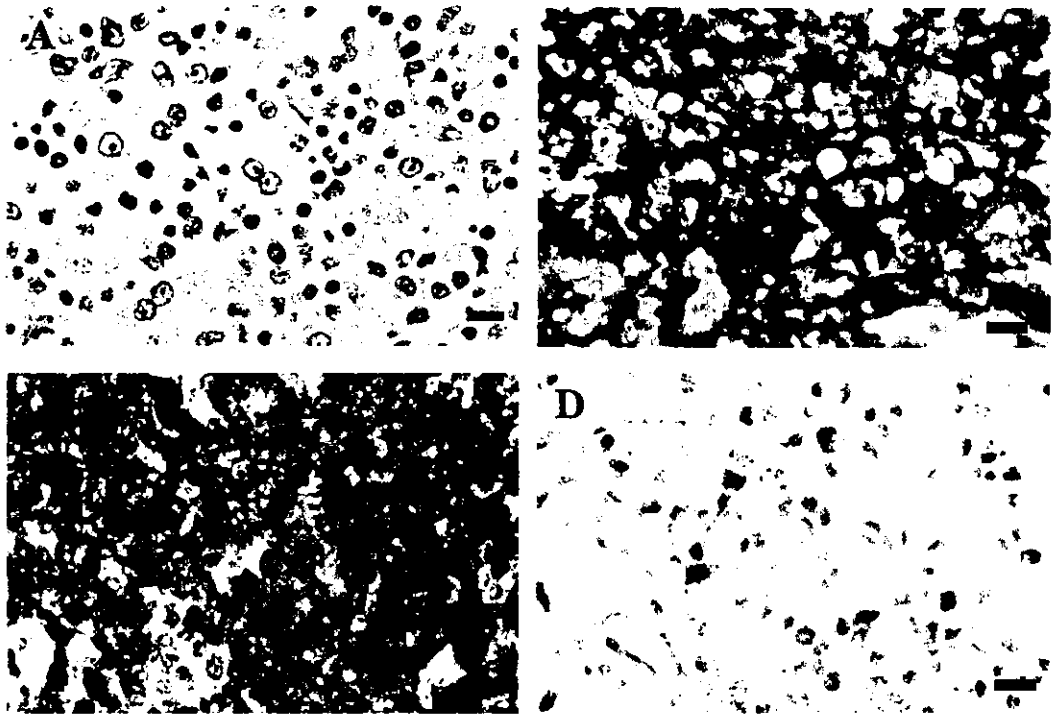

Fig. 1. Neoplastic lesion of the Japanese macaque. Bar = 10 μm. A; HE staining for the mediastinal lymph node. Hodgkin-like histiocytic and RS-like cells are observed. Mononuclear or binuclear RS-like cells are seen in the upper and the central parts. B; CD16+ neoplastic cells in the nasal lesion. Immunohistochemistry C; CD20+ neoplastic cells in the iliac lymph node. Immunohistochemistry. D; sEBER-positive neoplastic cells in the nasal lesion. *In situ* hybridization.

cluding axillary, mediastinal, gastric and iliac were as follows (Fig. 1A). Neoplastic histiocytic cells were proliferated diffusely. Multinucleated RS-like cells having eosinophilic and foamy cytoplasm with polymorphic or kidney-shaped nuclei were scattered in the lesion. Mirror-image in RS-like cells was often observed. Non-neoplastic lymphocytes were diffusely infiltrated into the lesion. Necrosis and fibrosis were apparent in part of the lesion. The lung, liver, spleen and the tumor mass in the left nasal cavity exhibited similar histopathological findings to those in lymph nodes. In the liver, neoplastic cells and non-neoplastic lymphocytes proliferated mainly at the Glisson's sheath.

The histiocytic neoplastic cells were positive for CD16 but negative for CD3 in any organs examined, while CD20-positive histiocytic neoplastic cells were recognized only in the iliac lymph node (Figs. 1B and 1C). RS-like cells were negative for CD3, CD16 and CD20.

Anti-STLV-I antibody was not detected in the case. The titers of anti-sVCA antibody were 1: 160 at 4.6 yrs. Simultaneously anti-sEA antibody was 1: 40. *In situ* hybridization revealed the expression of sEBER in the CD16-positive histiocytic neoplastic cells but not in the RS-like cells of all tissues examined (Table 1, Figs 1B and 1D).

Expression of sEBERs in the neoplastic cells and the concurrent presence of anti-sEA antibody suggest the important role(s) of sEBV in the lymphomagenesis in the present case. Infection of EBV in humans and sEBV in macaques is widely disseminated and causes B-cell malignancies such as Burkitt-type lymphoma. In addition, their frequent involvement even in Hodgkin lymphoma and the non B-cell lymphomas such as NK/T-cell lymphoma has been pointed out [4, 5].

Histopathological observations for the present case revealed that the neoplastic cells were composed of two types. One was round-shaped histiocytic cells (Hodgkin-like cells) with poor to rich cytoplasm and a dark or pale-stained nucleus, and the other was multi-

Table 1. Immunophenotype and sEBV related features of neoplastic cells

	Antibody to			Neoplastic cells* positive for			
	STLV-1	sVCA	sEA	sEBER	CD3	CD16	CD20
	-	1:160	1:40				
Tumor of nasal cavity				+	-	+**	-
Iliac lymph node				+	-	+	+
Liver				+	-	+	-
Spleen				+	-	+	-

*: RS-like cells were negative for all those markers. **: CD16-positive cells were identical to sEBERs-positive cells.

nuclear RS-like cells which are the typical cells of Hodgkin lymphoma in humans [7, 24] and some lymphomas of non-human primates [6, 20]. The ratio of the two cell types varied among the organs where the lesions were observed. Although morphological similarity to human Hodgkin lymphoma was observed in the present case, the majority of neoplastic cells were histiocytic cells with few inflammatory cells in the lesion. Hodgkin lymphoma in humans is characterized by a few RS cells with an inflammatory cell background [15]. These differences suggest that the present case is not comparable with Hodgkin lymphoma in humans.

One of the human non-Hodgkin lymphomas associated with EBV-infection, nasal type NK/T-cell lymphoma [15], manifests nasal involvement of the lesion, prolonged fever, anemia, body weight loss and a high alkaline phosphatase level. Histopathology of nasal type NK/T-cell human lymphoma is characterized by the proliferation of pleomorphic lymphoid cells with the occasional presence of Hodgkin/RS-like cells [27]. The phenotype of the lymphoma cells in the nasal lesion is EBER+, CD2+, CD3-, CD4+, CD8+, CD19-, CD30- and CD56+ [19, 29].

The present macaque case showed nasal involvement and a high alkaline phosphatase level and suffered from severe anemia. Phenotypes of the neoplastic histiocytic cells of the case were sEBER+, CD3-, CD16+ and CD20- in the nasal lesion, and sEBER+, CD3-, CD16+ and CD20+ in the iliac lymph node. For the detection of NK-cells, an antibody to human CD16 was used instead of anti-human CD56 antibody, since the reactivity of a selected anti-human CD56 antibody to monkey cells has not been confirmed. CD16 is also used as another marker to confirm this type of lymphoma [15, 19]. The appearance of CD20+ neoplastic NK/T-cells in the iliac lymph node corresponded to a previous observation in human T-cell lymphoma with an aberrant expression of CD20 and CD79a (both are B-cell markers) [32].

These comparable symptoms and laboratory findings suggest that the present case was a monkey type of nasal NK/T-cell lymphoma rather than Hodgkin lymphoma. The present case, in particular, is highly significant because the number of EBV-associated NK/T-cell lymphoma cases are increasing in humans. Further molecular analyses of the case may establish macaques as an animal model for EBV-associated T-cell or NK/T-cell lymphoma.

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ORIGINAL ARTICLE

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Changes in testicular and nipple volume related to age and seasonality in Japanese macaques (*Macaca fuscata*), especially in the pre- and post-pubertal periods

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Abstract We investigated, longitudinally and cross-sectionally, age and seasonal change in both the testis and nipple volume of Japanese macaques (*Macaca fuscata*) in relation to concentration profiles of gonadal steroids: testosterone (T) in males and progesterone (P) in females. Testicular volume (TV) and nipple volume (NV) showed rapid growth at puberty, 4.5 and 3.5 years of age in males and females, respectively, but in both sexes there were precocious individuals. The testis as a whole matures at about 10 years of age. TV change is closely related to T concentration profile. The pattern of TV change is composed of maturation and seasonal effects, with individual variation evident mainly in the latter. Some individuals show a simple pattern consisting of one peak in the breeding season (from summer to winter) and one trough in the non-breeding season. Other individuals exhibit a more complicated pattern composed of two or more peaks and troughs before and during the breeding season. The nipple matures at about 7 years but it is difficult to determine the exact maturational age as there are many confounding factors relating to NV. NV shows seasonal fluctuations similar to that of TV. Many animals have periods of substantial growth whereas others do not. The NV in adults from 10 to 25 years does not appear to change much with age, but animals older than 25 years of age have significantly smaller nipples. Seasonal fluctuation in NV mirrors that of the P level. Considered to be controlled by estrogen and P, the NV is a good indicator of the physiological status of reproduction, with its peak about 2 weeks earlier than that of P, that is, at the mid-follicular phase. NV and P level show a similar pattern in pregnancy; from conception, indicated by a P peak, NV and P concentration first decrease, then they increase until peri-parturition and slowly decrease again until the next breeding season.

Keywords Testis · Nipple · Puberty · Japanese macaques · Seasonality

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Introduction

Japanese macaques (*Macaca fuscata*) inhabit the Japan Islands, where a temperate climate prevails and four seasons are clearly demarcated. They show seasonality not only in body weight (Hazama 1964; Suzuki et al. 2000) and nutritional status (Hamada et al. 2003), but also in reproduction (Nozaki 1991). The breeding season is from autumn to winter (from September to February with a peak in November; Nozaki 1994), and the delivery season is from spring to early summer (from March to August with a peak in May; Nozaki 1994). Seasonality in the reproduction of Japanese macaques is predominantly controlled by day length, temperature, and internal calendar (Nozaki 1991). Similar seasonality has been reported in bonnet macaques (Glick 1979). Due to changes in their nutritional status or environmental conditions, female Japanese macaques are reported to experience their menstrual cycle in the non-breeding season (Mori et al. 1997). External genital organs change in size and/or color depending on the season: in males, the testis becomes larger in the breeding season (Nigi et al. 1980) and the skin on the scrotum, inguinal, peri-anal area, and caudal aspects of the thigh become reddish; in females,

the so-called "sexual skin" turns reddish and swells; and in both sexes the facial skin reddens.

Sexually dimorphic traits and their development in primates have attracted much attention (Dixson 1998), especially those in humans (Tanner 1962; Malina and Bouchard 1991). The reproduction-related characters mentioned above, comparable to the secondary sexual characters of humans, develop rapidly with the onset of puberty at about 3.5 years in females and 4.5 years in males (Hamada et al. 1999). In humans, facial, axillary, and pubic hair, breast development, testicular development, and voice changes (Tanner 1962) first occur in the adolescent period and rapidly develop. Some of these characters are also found in Japanese macaques, for example, testicular and musculoskeletal development in males or body proportion changes such as fat accumulation in females. Although the Japanese macaque differs markedly from humans in its strict seasonality in reproduction, it appears that the most striking difference is that women show no morphological changes associated with the menstrual cycle. Knowledge about what and how body part(s) change with age and reproductive maturity may shed light on the evolution of the reproductive system, especially as it relates to social structure. In view of this, the morphological changes associated with the reproductive state in Japanese macaques are of interest.

The purpose of this study was to examine age changes, seasonality, and changes in the physiological activity of reproduction in testis and nipple size (volume) in Japanese macaques. Although we have already preliminarily reported on size (Hamada et al. 1999), we extend analyses here with additional data to examine the relationship of changes in testis and nipple volume with the secretion of reproductive hormones, testosterone (T) for males and progesterone (P) for female. The testis produces sperm and secretes steroid hormones and its volume is considered to reflect the state of these functions (Nigi et al. 1980). Although there are some studies on testicular size variation in macaques (Sade 1964; Glick 1979; Nigi et al. 1980; Matsubayashi and Mochizuki 1982; Matsubayashi and Enomoto 1983; Hamada et al. 1996), the details of seasonal change and individual variation in testicular development have not been examined. The nipple appears to change due to growth, sexual maturation, aging, seasonality, and reproductive physiology (menstrual cycle and pregnancy) as mediated by several hormones and lactation. However, although the nipple has been studied from the perspective of behavior (nipple preference, e.g. Tanaka 1989), size variations have not been studied, except by Terasawa et al. (1983), who analyzed nipple size in rhesus macaques (*M. mulatta*) in the peri-pubertal period.

Methods

We adhered strictly to the "Guide for the Care and Use of Laboratory Primates" of the Primate Research Institute (2003), Kyoto University. Our research protocol was approved by the Animal Welfare and Animal Care Committee.

Subjects for longitudinal observation

We followed the growth and development of 15 subject macaques (5 males and 10 females) from the late juvenile (2 or 3 years) to the late adolescent (7 years) phase. Table 1 lists the dates of birth, periods of study, and numbers of observations. Although some females gave birth, almost all babies were separated from the mother at birth. The period of observation differed with the individual. Five macaques born in the same year were reared in the same group cage, composed of an outdoor (4×6×2.2 m, width × length × height) and an indoor enclosure (3.5×4×2.5 m). Monkey chow was the main food and supplements such as sweet potato or cabbage were given twice a week. The total caloric value of food was determined according to body weight and nutritional status, and water was accessible ad libitum. Somatometrical data were collected every 2 weeks. Subjects were anesthetized with a 2:1 mixture of ketamine hydrochloride (Ketalar 50, Sankyo Co., Ltd., 5 mg/kg body weight) and xyladine (Selactar 2%, Bayer Co. Ltd., 1 mg/kg body weight). Blood was collected by venipuncture every week. Somatometry and blood sampling were made from 11 a.m. after the subjects had fasted overnight.

Subjects of cross-sectional observation

Table 2 lists numbers of cross-sectional data by age and sex. We included longitudinal data points in this tabulation. Subject monkeys other than the longitudinal

Table 1 Subject macaques for the longitudinal measurements

No.	Date of birth	Age during study (years)	Number of observations
Male			
1412	1 June 1992	2.88–6.83	107
1428	25 May 1992	2.90–6.85	106
1439	5 June 1992	2.87–6.82	102
1459	12 March 1993	3.10–7.05	91
1470	5 May 1993	2.95–6.90	91
Total			497
Female			
1433	2 June 1992	4.28–6.83	63
1447	23 June 1992	4.14–6.77	62
1458	31 March 1993	3.47–7.00	81
1460	27 March 1993	3.48–7.01	81
1469	30 April 1993	3.39–6.92	82
1720	2 May 1998	1.95–5.10	68
1729	28 May 1998	1.88–5.02	74
1736	29 May 1998	1.88–5.02	72
1743	9 June 1998	1.85–4.99	73
1744	10 June 1998	1.84–4.99	73
Total			729

Table 2 Number of measurements with age and sex: cross-sectional data

Age (years)	Male	Female	Total
0	3		3
1	9	24	33
2	24	121	145
3	150	123	273
4	137	169	306
5	131	134	265
6	107	104	211
7	10	12	22
8	6	10	16
9–10	6	14	20
11–15	19	24	43
15–20	12	25	37
20–25	12	31	43
25–		14	14
Total	626	805	1,431

subjects were reared in open enclosures of about 800 m^2. During the annual health inspections, which were carried out in autumn, we collected somatometric data and blood samples. We also added data collected arbitrarily in other seasons.

Somatometry

The length (or height, L in millimeters) and width (or diameter, W in millimeters) of the right testis and nipple were measured to the nearest millimeter with a sliding caliper. Assuming an ellipsoid shape for the testis and a cylindrical column for the nipple, we calculated their volumes as follows: testicular volume (TV, milliliters) = $W^2 \times L \times \pi/6{,}000$, in which π stands for the circular constant (Marson et al. 1991), and nipple volume (NV, milliliters) = $W^2 \times L \times \pi/4{,}000$.

Measurement of the concentration of gonadal steroid hormones

We measured the plasma concentration of gonadal steroid hormones, T for males and P for females, to monitor the physiological states of reproduction. In humans, the T level varies within a day with its peak and trough concentrations found in the morning and evening, respectively (Felig et al. 1995). We followed the recommendation that blood be collected at the same time each day (Felig et al. 1995) and sampled animals between 11:00 and 11:30 a.m.

We analyzed the concentration profile of P in the luteal phase to determine the ovulatory menstrual cycle (Nozaki 1991). Although puberty is often determined by menarche, the first menstrual bleeding, as subjects were reared in group cages, that method was not applicable. Reproductive maturation can also be determined by ovulation but the determination of this by direct observation of the ovary requires invasive surgery and anesthesia (Terasawa et al. 1983). The menstrual cycle is often depicted by changes in the concentrations of serum E2 (17β-estradiol), luteinizing hormone (LH), follicle stimulating hormone (FSH), and P. Thus, one of these hormones can represent the cycle in cases where reproductive cycles occur regularly. Although estrogen is known to stimulate breast development at puberty in girls (Mastroianni and Coutifaris 1990), and the development of nipple size in rhesus monkeys is controlled by E2 (Terasawa et al. 1983), estrogen is secreted in a pulse-like surge and high levels occur for only 48 h during mid-cycle (Johnson and Everitt 1995), so frequent sampling is necessary. The secretion patterns of LH and FSH are similarly pulse-like. The period of secretion of P, however, continues for much longer and P of higher than 1 ng/ml is secreted for more than 1 week (in rhesus macaques, Knobil and Hotchkiss 1988; in Japanese macaques, Nozaki 1991). The concentration of P also indicates the maturational status of the ovary because P is secreted directly from here. Therefore, P was regarded as a more appropriate indicator for the present study in which blood samples were collected weekly. Concentrations of plasma T and P were measured with standard radioimmunoassay kits (for details see Suzuki et al. 2000).

Statistical analyses

We examined the age change (maturation) and seasonality of TV and NV, and hormonal profiles, mainly graphically but also with basic statistics using Excel (MicroSoft) and S-Plus 4 (MathSoft). We occasionally smoothed graphs using the loess.smooth function (S-Plus 4, MathSoft). Age was expressed to the order of 0.01 years for analyses, and age and calendar month were used to examine seasonal influences. As the birth dates of subject monkeys were in spring, (range from 12 March to 23 June, average 14 May, see Table 1), age and calendar month and age in years are interchangeable (e.g. the age in years for November at 4 years is about 4.5 years old).

Results

Age change and seasonality in testicular volume (TV)

All cross-sectional data on TV were plotted against age (Fig. 1). TV started its rapid increase at puberty, at about 4.5 years, and matured when the individual became an "adult" at 7 years on average, but there was variation with locality (Nigi et al. 1980; Hamada et al. 1986). Although the variation was considerable after puberty, the maturation pattern of the testis is well depicted by the diagram. In adolescence, considerable seasonal fluctuation was observed. For adults (≥7 years), the median is 42.2 ml, and TV for the 10th and 90th percentiles was 23.9 and 65.8 ml, respectively (Fig. 1a). In the range of TV up to 7 years of age, the maximum was close to the 90th percentile of adults, but the mini-

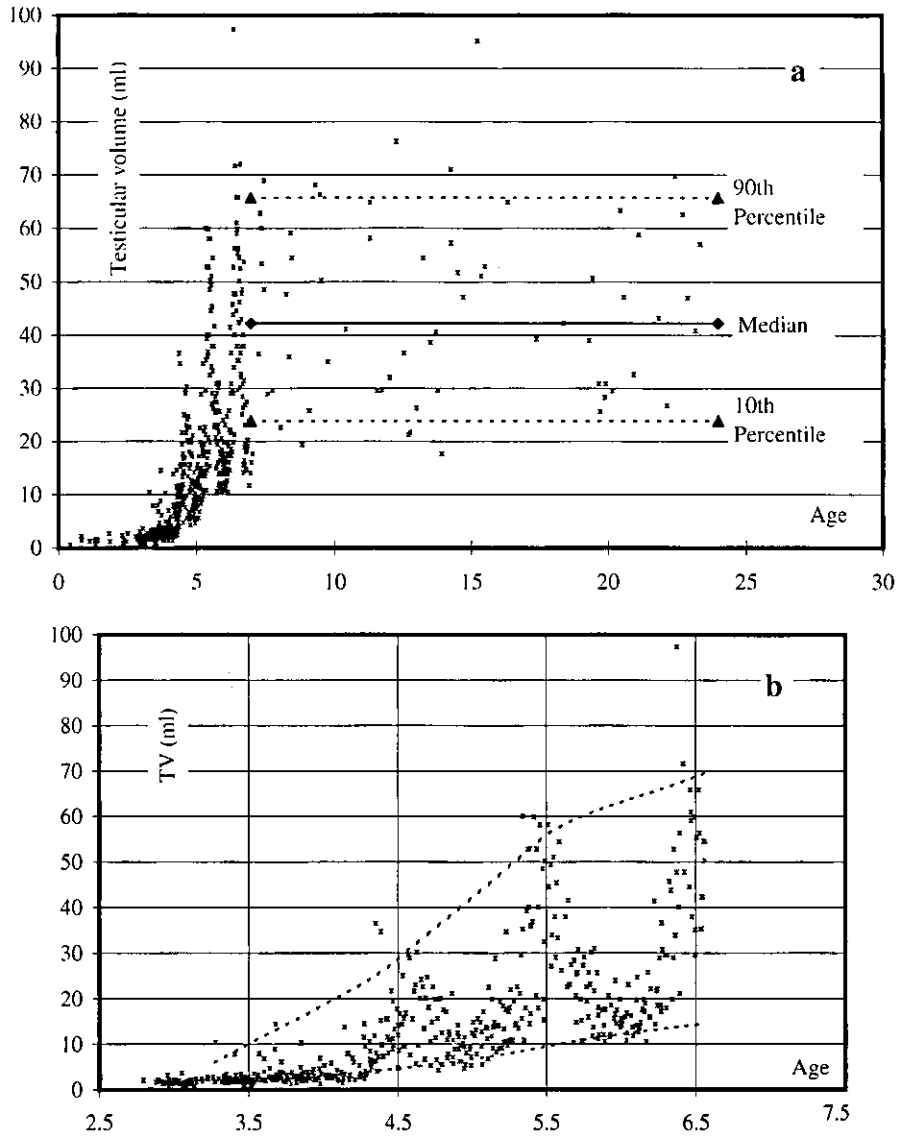


Fig. 1a, b Plots of testicular volume (TV) against age in Japanese macaques. **a** In the adult period, 7–25 years, the median (*line* and *diamonds* at both ends), 10th and 90th percentiles (*dotted lines* and *triangle* at both ends) are superimposed on the diagram. **b** Detail from 2.5 to 7.0 years of age. *Smoothed curves* Connecting peaks and troughs are superimposed

mum (baseline) was smaller than the 10th percentile of adults (Fig. 1b). Therefore, the testis did not appear fully mature at 7 years but it did by about 10 years of age. The oldest monkeys from which we could collect TV measurements were 24 years but we did not note any significant change of TV with age in adults.

Figure 1b shows the details of TV change from the late juvenile to the adolescent period, that is, 3–7 years. TV increased gradually from 3.0 to 4.2 years of age. Although some individuals exhibited a much greater TV than the majority at about 3.5 years (breeding season), a seasonal influence was noted even in these precocious individuals. The TV rapidly increased from 4.2 to 4.6 years (breeding season), to produce the first peak in the graph, and then rapidly decreased to produce a trough at about 5 years (delivery season). Notably, the volume at the trough, the baseline volume (10.36 ml $\pm$3.39 SD, $n=28$, for subjects aged from 4.90 to 5.10 years), was significantly greater than that at about 4 years of age (3.40 ml $\pm$1.39 SD, $n=24$, for subjects aged from 3.90 to 4.10 years; $P<0.01$ by t-test).

The testis increased again from the baseline at 5.0 years and achieved a peak of 40–60 ml in the breeding season, whereupon it rapidly decreased again to the trough at about 6.0 years of age in the delivery season. The baseline volume at that age was approximately 15 ml, about 5 ml greater than the previous trough. From 6 to 7 years of age, this seasonal cycle was repeated with greater amplitude, and the baseline volume increased further.

Age changes in TV in the five individuals observed longitudinally are shown in Fig. 2 where the abscissa represents age and calendar month. This graph shows that TV change with age was composed both of seasonal increases and decreases and overall growth. The amplitude of the seasonal cyclical change became greater with age. Because the cyclicity of all individuals was fairly well synchronized, the age change analyzed cross-sec-

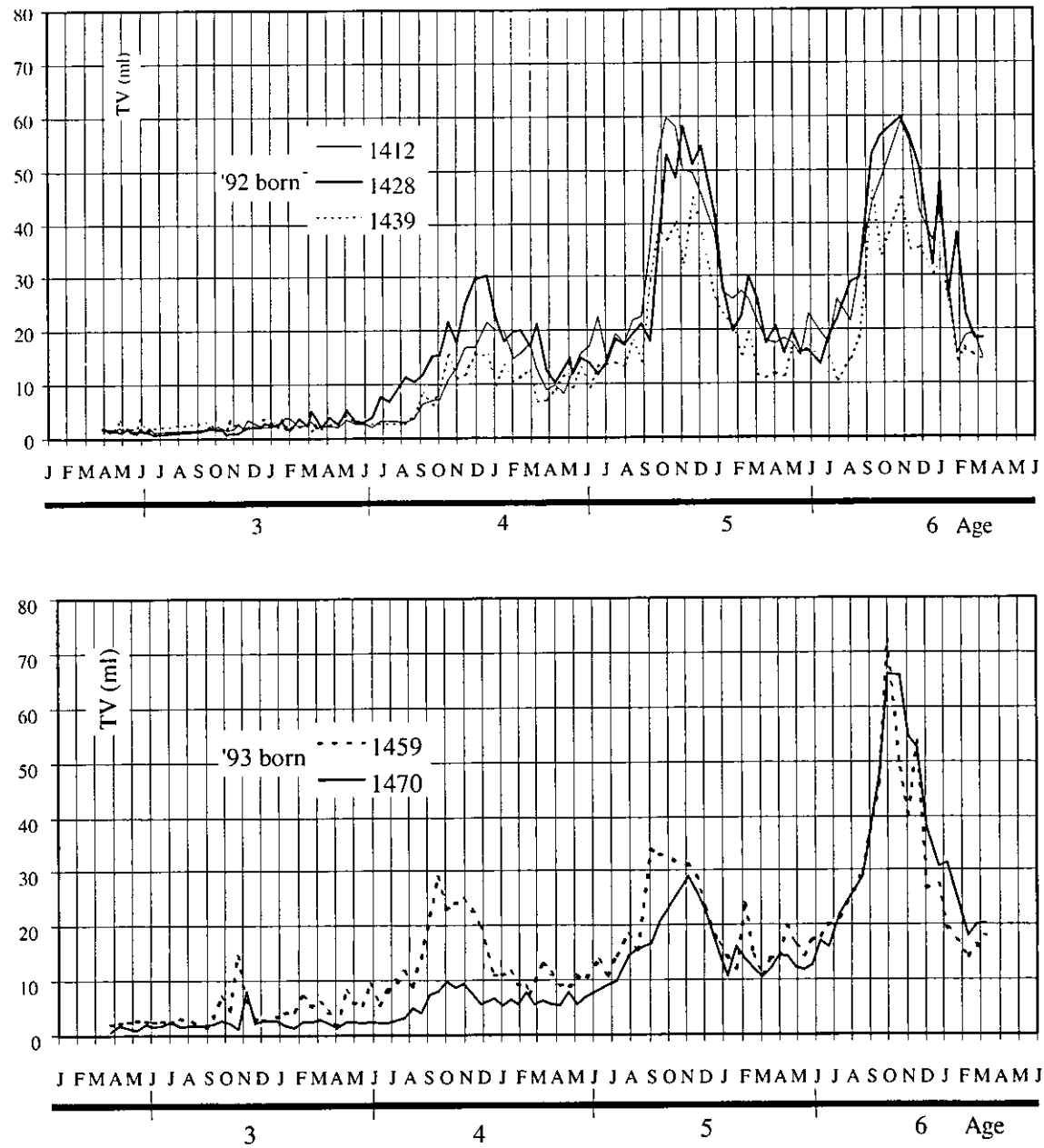


Fig. 2 Longitudinal age change of TV in five males, from 3 to 7 years of age, *top* for subjects born in 1992 and *bottom* for those born in 1993. *Abscissa* is the age and calendar month

tionally preserves the seasonal changes (see Fig. 1b). The dates at peak TV, however, differed somewhat between individuals. Two individuals, numbers 1459 and 1470, showed clear but small peaks in October of the year they were 3, indicating that they were precocious. Before 7 years, their ages at peak TV were slightly younger than those of the other three individuals. Thereafter, the ages of peak TV were similar for all five individuals.

In every individual, peak TV occurred between October and December and then the volume rapidly decreased to its baseline around April. The TV again gradually increased from April, but from mid-September the velocity of increase accelerated until peak TV was reached.

Figure 3 shows the seasonal change of TV in adults of more than 7 years of age, but note that data were not available for every month. As shown by the polynomial regression curve, TV decreased from February to May or June and then it increased in autumn and winter. The maximum volume is about twice that of the minimum.

Testosterone secretion and TV: from the longitudinal analysis of the peri-adolescent period

Figure 4 depicts two examples showing typical relationships between the concentration of serum T (nanograms per milliliter) and TV. Individual 1412 (Fig. 4a)

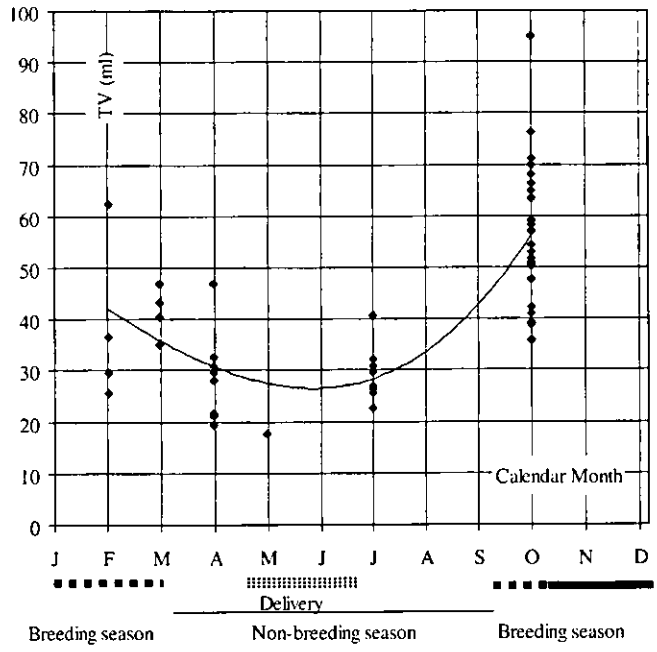


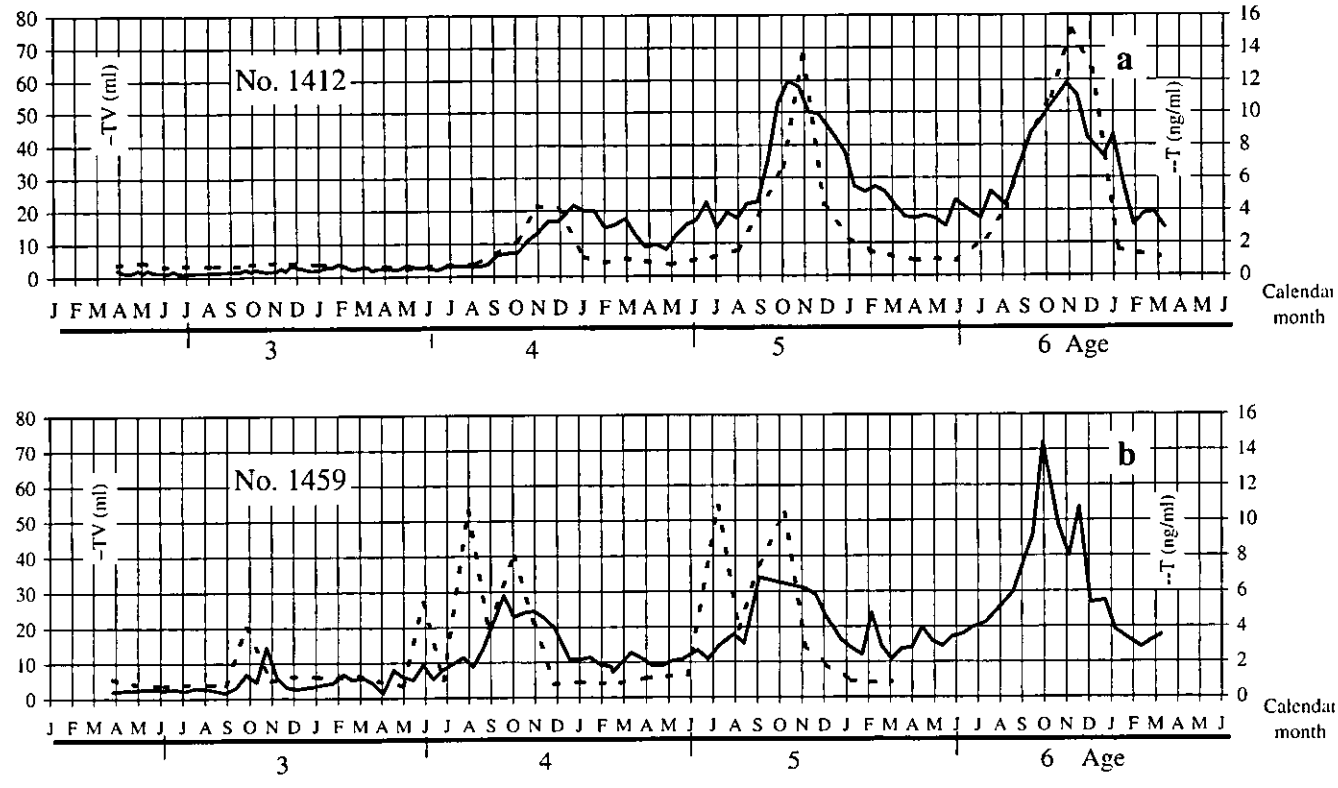
Fig. 3 Seasonal change of TV in adults

shows a simple cyclical pattern with one peak and trough in secretion each year starting from the winter (breeding season) of 4 years of age (ca. 4.5 years). The peak T concentration increased with age: around 4.2 ng/ml at about 4.5 years, 13.5 ng/ml at about 5.5 years, and 14.5 ng/ml at about 6.5 years. The T concentration started to increase rapidly in summer (June–September) and its decrease from the peak concentration was similarly rapid to reach baseline in January or February. The change in TV synchronized well with the T concentration profile, except for the first TV peak, which was later than the T peak by about 1.5 months. TV also started to increase rapidly from 4.5 years, which was concurrent with a rapid increase in T concentration. TV attained its adult mean volume at about 5.5 years of age.

In individual 1459 (Fig. 4b), T secretion occurred early in the year, in both summer and autumn, and there was more than one peak of secretion. At 3 years of age, this individual was clearly precocious to have experienced a significant peak in T secretion in October, with a peak concentration of 4.0 ng/ml, which is comparable to the first peak concentration in individual 1412 at about 4.5 years. Notably, TV temporarily increased in November. At 5 years, there were three peaks in T level, in June, August, and October, the highest of which occurred in August. TV gradually increased from June to August and then, from September, it rapidly increased to the maximum volume of this age, around 30 ml. Although the volume gradually decreased from its peak, it was still more than 20 ml at the end of December. The TV decreased to its baseline of about 10 ml in January and this volume was maintained through to July at 5 years of age.

In the breeding season at 5 years, there were two T peaks at the beginning of July and October, and the TV

Fig. 4a, b Two typical examples showing the relationship between testosterone (*T*) secretion (*dotted line*) and TV change (*solid line*). **a** Simple pattern. **b** Complicated pattern showing secretion in summer



attained a peak volume of around 35 ml, smaller than 40 ml, the median of adults. As TV matured only in the following breeding season, TV development in this individual was retarded. There was a lag (latency period) between T and TV in this individual in the breeding seasons from 3 to 5 years of age when the TV had not yet matured. Of interest is that monkeys reared in the same cage tended to exhibit similar seasonal patterns of TV and T level, that is, numbers 1428 and 1439 were similar to number 1412 and number 1470 was similar to number 1459.

Age change and seasonality of nipple volume

Nipple volume (NV) did not change between birth and 2.5 years (Fig. 5). The nipples grew rapidly from the breeding season of 3 years and matured either in the latter half of adolescence or in the young-adult stage (5–10 years of age). Basic statistics for adults (aged 10–25 years) were mean 4.82 ml (SD = 3.60), median 3.21 ml, 10th and 90th percentiles 0.66 and 8.83 ml, respectively. Thus, the NV in adults was highly variable (Fig. 5a). In adults, there was no significant change in NV with age. Older individuals (≥25 years of age), however, tended to have smaller nipples, with an average of 1.18 ml (SD = 0.96). The difference between means was significant ($P < 0.01$, t-test).

Figure 5b focuses on animals younger than 10 years. The nipple grows slowly from birth to 4 years, to reach a maximum of 1.0 ml. Although some data points are larger than 2.0 ml, the majority fell within the range of 0.5–1.0 ml. A seasonal fluctuation in NV was not evident from this diagram.

We examined the longitudinal age changes in NV in the juvenile and adolescent periods, from 2 to 7 years of age (Fig. 6). With the exception of individual 1447, the graphs of the other nine animals displayed similar age and seasonal changes. The exceptional subject, number 1447, had large nipples that fluctuated unusually. Subject 1729 was precocious by about 6 months. Our subject females first gave birth between 5 and 6 years of age. The distortions to cyclicity caused by pregnancy and lactation are described later.

Change of NV in early adolescence is represented by the graphs of two typical examples (Fig. 7). Individual 1460 (Fig. 7a) displayed regular cyclicity, with almost the same heights of peak NV and the same amplitude of seasonal fluctuation at 3, 4, and 5 years of age.

Subject 1744's (Fig. 7b) peak NV increased greatly from the fourth to the fifth winter, almost doubling in size, and a seasonal change was also evident. Many of the other subjects exhibited trajectories similar to that of 1744, where the baseline volume increased from 4 years and the growth component was quite large. In the fifth winter, there were two additional peaks in NV both before and after the peak in January, and a NV of greater than 0.8 ml was maintained for nearly 6 months

The relationship between the P concentration profile and NV change

Figure 8 shows the P concentration profile and NV change in early adolescence (no. 1469). The first signif-

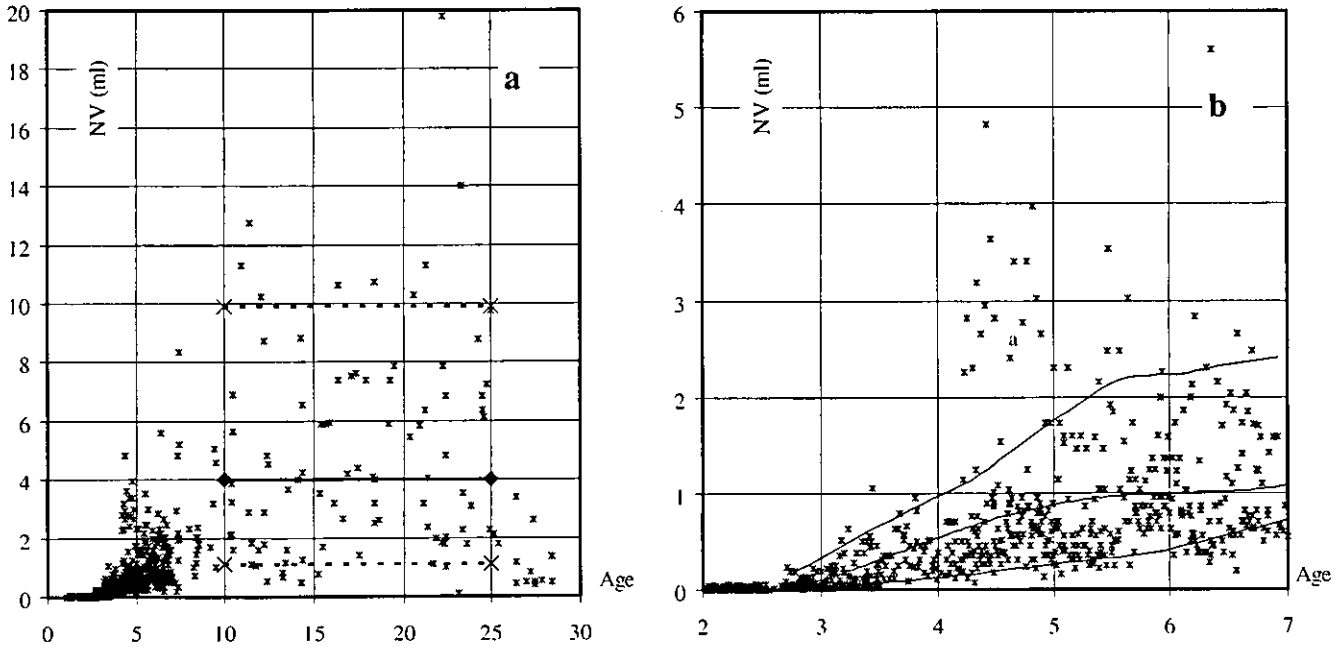


Fig. 5a, b Plots of nipple volume (*NV*) against age in female Japanese macaques. **a** In adults from 10 to 25 years of age, median (*line* and *diamonds* at both ends), 10th and 90th percentiles (*dotted lines* and *X* at both ends) are superimposed. **b** Nipple change from juvenile to young adult stages. Smoothed curves connecting peaks and troughs are superimposed

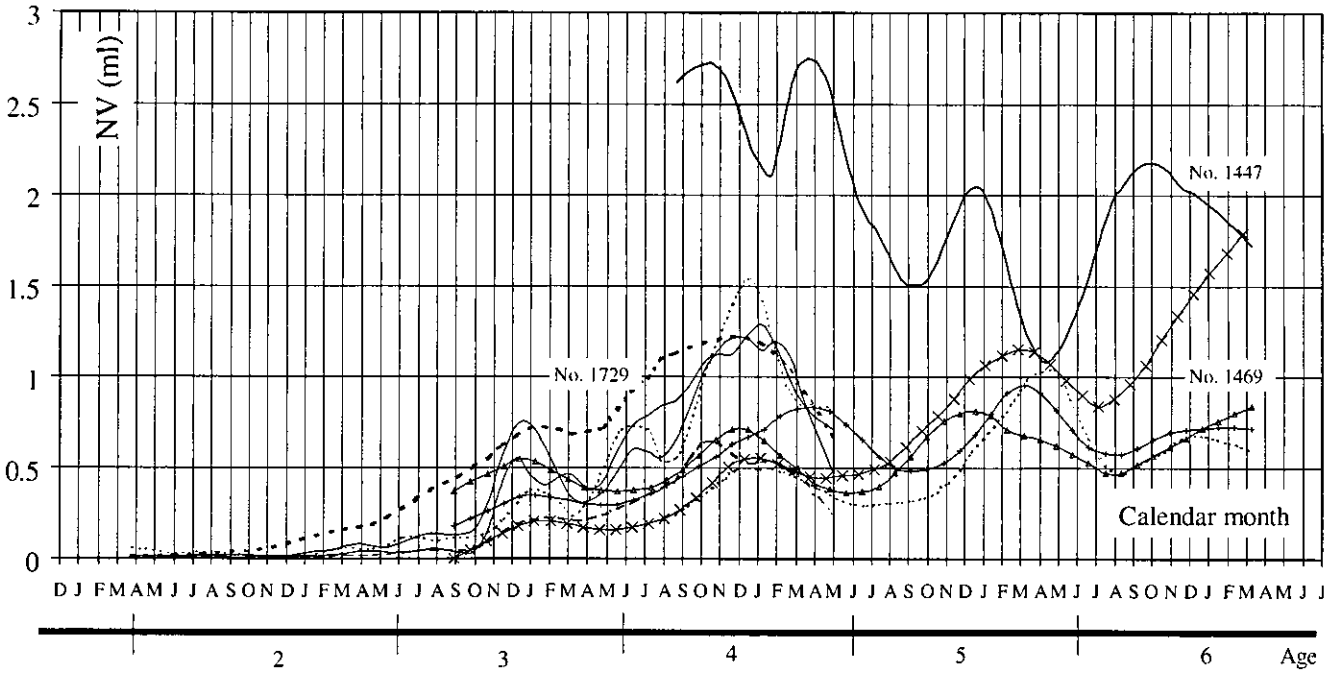


Fig. 6 Longitudinal age change of NV in ten females from 2 to 7 years of age. *Abscissa* is the age and calendar month. The *thick line*, *dotted thick line*, and *line with X* are the plots for subjects 1447, 1729, and 1469, respectively

icant P peak was found as late as 30 January at 3 years. There was only one peak in that season and NV increased to attain only a low peak, which occurred a little earlier than the P peak.

In the next breeding season, the P level had two major peaks, on 20 November and 18 December, and a smaller peak, little more than 1 ng/ml, occurred on 8 January. The NV started to increase from November and attained its peak in February. Although NV decreased from that peak to a trough in March, it had increased again, despite fluctuations, to 0.6 ml on 30 July. The increase in summer was not accompanied by P-level elevation.

In the third breeding season (at 5 years of age), the P level was slightly higher than 1 ng/ml in October, and then it suddenly increased to its peak on 12 November. We believe that the individual conceived around that date. The NV decreased in early autumn until the 3rd of September, but then it started to increase until around the time of conception.

We next examined the relationship between the concentration profile of P and change in NV in two examples from middle to late adolescence, that is, from 4 to 6 years of age (Fig. 9). Individual 1433 (Fig. 9a) experienced three P peaks in the winter when she was 4 years of age (5 December, 9 January, and 6 February), and following these, a lower peak was found on 14 March. In that same breeding season, we observed three peaks in NV on 20 November, 22 January, and 20 February. After the measurement on 18 December, we ought to have taken measurements on 1 January, but we could do so only on 8 January. If we suppose that a peak occurred there, then a peak in P followed a peak in NV by a reasonably regular lag of about 2 weeks.

The next cycle was initiated in November, and the subject conceived at around the time of the first P peak. The NV and P concentration profile followed the pregnancy pattern that is outlined below.

Although subject 1447 was previously described as an exceptional, she did show another typical pattern of P secretion and NV change (Fig. 9b). In this animal's fourth year, NV increased with three peaks on 24 September, 23 October, and 4 December. The P peaks, however, occurred on 12 December and 9 January. At around the time of the first P peak, the subject was considered to have conceived. NV and P levels showed great fluctuations during pregnancy, and the increase in NV in autumn was not accompanied by a P-level increase.

After parturition, P concentration remained below detection level until the middle of November and then four peaks occurred on 4 and 25 December, 22 January, and 19 February. The NV also peaked four times at roughly monthly intervals, on 13 November, 10 December, 15 January, and 12 February. Considering the interval of measurement of both P concentration and NV, a 2-week lag appears reasonable. The average NV over the 4-month breeding season was about 1.80 ml, higher than the baseline volume of about 1.0 ml. The NV increased again from July to attain a peak on 9 September. Thus, NV increased from summer to autumn as it had when the animal was 4 years old. The P peak was found on 26 November and 24 December and the subject conceived at around the time of this second peak.

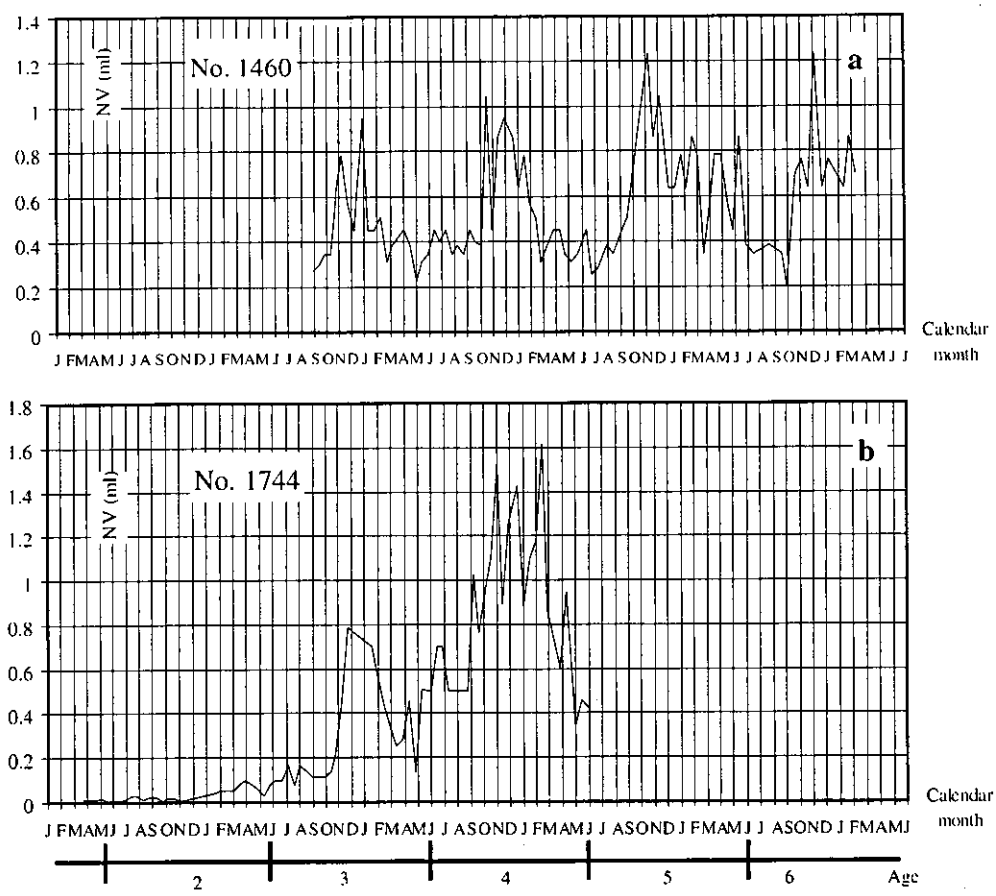


Fig. 7a, b Two examples showing the age change pattern of NV **a** for number 1460: a simple cyclic pattern where the baseline volume does not change with age, and **b** for number 1744: a pattern showing seasonal cyclicity and growth

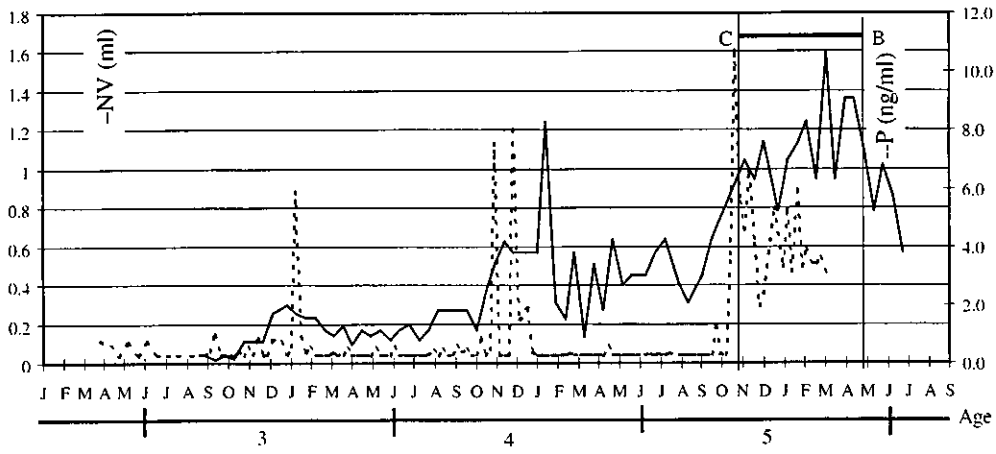


Fig. 8 Age change of NV (*solid line*) and the secretion profile of progesterone (*P*, *dotted line*) in a female subject from the juvenile to the middle of her adolescent period. At 5 years of age this subject became pregnant. In the diagram, *C* (estimated conception), *B* (parturition), and the *thick line* (pregnancy) delineate the pregnancy-related changes

The influence of reproduction on NV: pregnancy pattern

NV change over the period of pregnancy was examined using the P concentration profile for the determination of the day of conception (see Suzuki et al. 2000). The subject depicted in Fig. 8 exhibits the general trend. She conceived in November at 5 years of age and, at that time, NV was already on the increase, as it had been since summer. In many cases, the NV did not show any outstanding characteristics such as a peak or trough on the day of conception. From the P concentration profile, we determined that many subjects conceived during the first or second peak of concentration.

After conception, although NV temporarily decreased, it gradually increased, despite fluctuations, with the advance of pregnancy (see the latter half of 5 years in Fig. 9a and the latter half of 4 years in Fig. 9b). The subject delivered around the time when NV had attained its maximum or second maximum peak in the birth season. After parturition, NV decreased slowly from spring to autumn with fluctuation until it started to follow the menstrual cycle. The concentration profile of

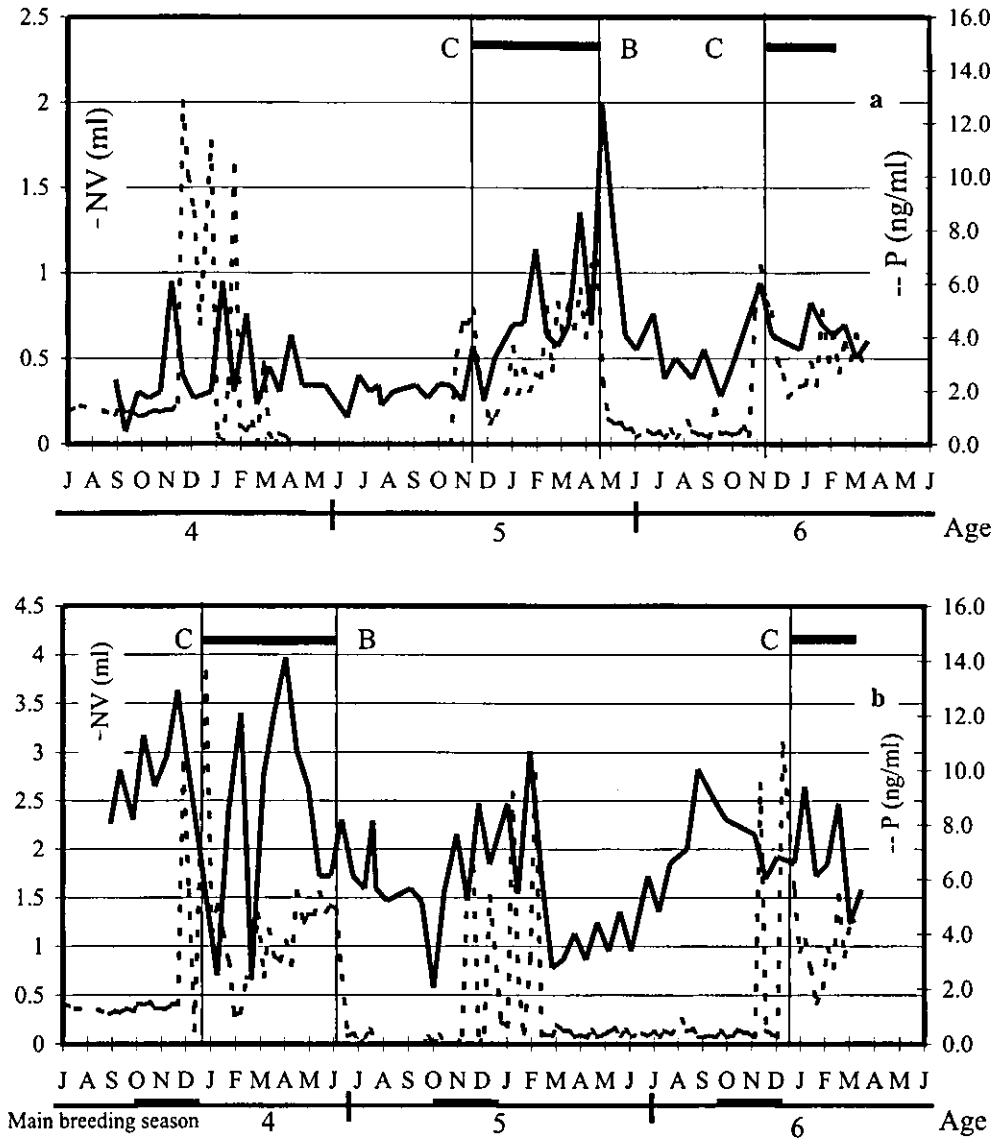


Fig. 9a, b Two examples showing the relationship between the age changes in NV (*solid line*) and the secretion profile of progesterone (*dotted line*). **a** Number 1433; **b** number 1447. *C* (estimated conception), *B* (parturition), and the *thick line* (pregnancy) delineate pregnancy-related changes

P followed almost exactly the change in NV from conception to parturition, but the secretion of P ceased from just after parturition until the next breeding season.

The infant of subject 1469 suckled from its mother for more than 1 year after birth, and the NV graph of this subject increased substantially (Fig. 6). In contrast, the other four subjects in Fig. 6 exhibited the same cycle in the 7th year of life as that found in the previous year. Thus, although NV appeared to increase due to suckling, the exact effect of suckling, especially the cumulative effect, on NV is left for future study.

Discussion

Development and seasonality of the testis in Japanese macaques

The outline of testicular development in Japanese macaques is as follows: the testis starts its rapid increase in size at puberty during the breeding season when animals are about 4.5 years old (e.g. Nigi et al. 1980; Matsubayashi and Mochizuki 1982). There is considerable variation with locality (Hamada et al. 1986) and nutritional status (Hamada et al. 1999) but, on the whole, the testis matures at about 10 years of age (Matsubayashi and Mochizuki 1982). Although the number of subjects was limited, TV did not show age-related changes after reaching maturation. Some animals that grew faster than average in physical dimensions (e.g., body weight) were precocious and showed a small temporal TV increase 1 year earlier. Male long-tailed macaques show a tendency of "catch-up" testicular development, that is, the later the testis starts pubertal development, the faster it develops (Meussy-Dessolle and Dang 1985). We did not, however, find any such significant "catch-up" in the present study.

The TV in Japanese macaques shows a strict seasonality, with an increase before and during the breeding season and a rapid decrease at the end of the season. The

first TV peak at puberty is significantly smaller than those that occur thereafter. The amplitude of seasonal cyclicity and baseline volume becomes higher as animals mature. The concentration of T increases from 4 to 7 years with considerable fluctuation due to seasonality. Individual variation is substantial in terms of age at T peak and TV peak but, as the cyclicity and amplitude correspond closely among individuals, the cross-sectional diagram preserves the seasonal effect (Fig. 1b).

The relationship between TV and T level was quite tight in each of the individuals, but there was a slight lag between T peak and TV increase in the period where TV had not yet attained maturity (see Fig. 4). Matsubayashi and Enomoto (1983) reported for adult Japanese macaques that the T level increases rapidly from July to attain a peak in September, and that the TV peak is found in October. Their result appears to show a lag between T secretion and TV increase, but this may reflect their measurement schedule. Whereas they measured T monthly, TV was measured only four times a year (April, July, October, and January). The time lag between TV increase and T secretion in immature individuals is quite different from that between the frequency of breeding behaviors (mounts) and T secretion (Rostal et al. 1986).

The presence of cycling females has been reported to influence males sharing the same environment (Vandenbergh 1969). We found that males in the same cage tended to show similar profiles, and, notably, cycling females were also reared in these cages. We could not, however, substantiate any such influence in this study (see pp 389–392 in Dixson 1998).

Development and seasonality in the nipple size of Japanese macaques

Nipple size variation in macaques has not attracted much attention, though there are studies that deal with nipple preference (laterality, e.g. Tanaka 1989). The size varies widely among individuals because there are many factors influencing it: growth, reproductive maturation, menstrual cycle, physical factors such as suckling by offspring, pregnancy and parturition, lactation, and aging. In the peri-adolescent period, the age change in nipple size appears consistent.

The nipple in Japanese macaques starts to grow rapidly at about 3.5 years of age, in conjunction with other characters that advertise reproductive state (the swelling of sexual skin and reddening of the face and sexual skin). The Japanese macaque subjects showed substantial P secretion in their fourth winter (ca. 3.5 years of age), indicating that they may have commenced their menstruation cycles, and their NV increased accordingly over the fourth summer and autumn. Therefore, it seems that many of them experienced menarche in the autumn and, a few months later, their first ovulation. In the case of rhesus macaques (Terasawa et al. 1983), the period between menarche (30.7 ± 1.2 SE months of age) and first ovulation (48.1 ± 2.2 SE months of age) is much longer than that in Japanese macaques.

The nipples finally mature at around 7 years of age in Japanese macaques (5–10 years, the latter half of adolescence and the young-adult period). Seasonality in NV was evident in every female analyzed longitudinally where there was a peak in the breeding season (winter) and a trough in the delivery season (spring). In contrast to the testis, seasonality in NV, starting from puberty, was not evident in the cross-sectional data analysis because of wide individual variation. Throughout the adult period, from 10 to 25 years of age, NV seems to be consistent, though there may be seasonal fluctuation and a cumulative effect of being suckled by offspring in adult macaques, but these are topics for future study. In general, NV gradually decreases from 25 years of age, when, on average, the post-menopausal period begins (Takahata et al. 1995; Pavelka and Fedigan 1999).

The NV in many individuals correlates strongly with changes in reproductive physiology as represented by the P concentration profile in this study. Thus the NV follows the menstrual cycle, and its peak precedes the P peak by about 2 weeks (the mid-follicular phase). The menstrual cycle is often characterized by the concentration profiles of E2, gonadotropins (LH and FSH), and P (Dixson 1998), and the fact that the NV peak is found in the mid-follicular phase indicates that E2 is the major hormone influencing nipple enlargement. A close relationship has been reported between the E2 level and nipple size in rhesus macaques (Terasawa et al. 1983). Nevertheless, there also exists the possibility that other hormones are also involved.

The combination effect of P and E2 on nipples is worth considering even though Terasawa et al. (1983) found no relationship between P and NV before ovulation in the peri-pubertal phase. In the luteal phase P is secreted mainly in preparation for pregnancy (e.g. synthesis of secretory material by uterine glands, Johnson and Everitt 1995; cellular differentiation in the uterus, Baulieu 1992), and both E2 and P are considered to function in maintaining the increase of NV. In the breeding season, when several menstrual cycles occur, the average NV remains higher than that in the non-breeding season. This difference may be due to the fact that the regular secretion of many hormones, including E2 and P, keeps the nipples large throughout the breeding season. The combination effect of P and E2 is also found in the change of NV during pregnancy. After conception, both P and E2 are secreted and the change in NV parallels the concentration profile of P. It is known that P is an antagonist of mineralocorticoids, causing water retention (Felig et al. 1995). The two hormones, E2 and P, in conjunction with others, influence the breasts of women in the luteal phase (Johnson and Everitt 1995), so their combined effect may influence the nipples in Japanese macaques.

The other pattern of NV change that must be explained is that NV increased in some subjects in the

non-breeding season, from spring to autumn. We suspected that a non-ovulatory menstrual cycle caused this increase. Such cycles have been reported for subjects reared in indoor cages with a relatively high frequency (Nozaki 1991). However, as the subjects for the present study were reared in a cage with an outdoor enclosure, it seems an unlikely explanation. Mori et al. (1997) reported on food-enhanced perineal swelling in Japanese macaques in poor nutritional condition, but, although reproductive state is known to relate closely to nutritional condition (Nigi et al. 1995; Nigi and Morimitsu 1997), it could not be used as an explanation in this case. This, then, is also a subject for future study.

The relationship between NV change and social life

It is widely known that Japanese macaques have a multimale, multi-female type of society and will not make "fission and fusion" as chimpanzees do (e.g. Melnick and Pearl 1987), and they are always attentive to the behavior of other members, such as posture and all kinds of facial and tail movements, however slight. It follows then, that they probably also recognize morphological changes in other individuals. The morphological changes related to reproduction, such as sexual swelling, reddening of the facial and sexual skins, and enlargement of testes and nipples, would function as visual cues. Although NV varies considerably among individuals, the present study showed that changes in a given individual due to seasonality and the menstrual cycle are great enough to be detected by other troop members. Humans familiar with subject macaques can also detect the change. Japanese macaques tend to sit keeping their torsos erect, and this posture ensures that the nipples are visible to other members. Nipples appear to advertise secondarily the reproductive state of an individual, together with the remarkable advertisement found in the anogenital region and the caudal aspects of the thigh that are obscured when the individual sits. The present study suggests that peak NV occurs in the midfollicular phase, that is, about 1 week earlier than ovulation. This pattern is similar to that of the sexual swelling in chacma baboons (Bielert 1986), where the skin swells in the follicular phase and starts to break down around the day of ovulation, and frequent ejaculate is observed around the late follicular phase. As Japanese macaques have keen vision, and reddish nipples contrast sharply against their whitish chest, other troop members can detect the nipple change and respond appropriately.

Nipple size has often been used by human observers to classify adult females as either parous or nulliparous for the determination of population structure with considerable accuracy (H. Ohsawa, personal communications). It is also possible for human observers to determine whether the individual is lactating or not simply by observing the nipples. It is, therefore, thought that macaques can also discriminate that difference. We have yet, however, to elucidate the exact relationship between size changes in nipples and behavioral changes in other individuals.

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JNK promotes Bax translocation to mitochondria through phosphorylation of 14-3-3 proteins

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Targeted gene disruption studies have established that the c-Jun NH_2-terminal kinase (JNK) is required for the stress-induced release of mitochondrial cytochrome *c* and apoptosis, and that the Bax subfamily of Bcl-2-related proteins is essential for JNK-dependent apoptosis. However, the mechanism by which JNK regulates Bax has remained unsolved. Here we demonstrate that activated JNK promotes Bax translocation to mitochondria through phosphorylation of 14-3-3, a cytoplasmic anchor of Bax. Phosphorylation of 14-3-3 led to dissociation of Bax from this protein. Expression of phosphorylation-defective mutants of 14-3-3 blocked JNK-induced Bax translocation to mitochondria, cytochrome *c* release and apoptosis. Collectively, these results have revealed a key mechanism of Bax regulation in stress-induced apoptosis.

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Introduction

Apoptosis is essential for normal development and maintenance of tissue homeostasis. Disruption of the equilibrium between pro- and anti-apoptotic factors results in pathological conditions such as cancer, autoimmune disease and neurodegenerative disorders (Krammer, 2000; Yuan and Yankner, 2000). The loss of mitochondrial membrane integrity and the consequent release of cytochrome *c* into the cytosol are important events during apoptosis and are regulated by the Bcl-2 family of proteins (Wang, 2001). As the members of the Bcl-2 family exert their actions mostly at the level of mitochondria and reside upstream of the onset of irreversible cellular damage, they play a pivotal role in determining whether a cell will live or die (Gross *et al*, 1999; Tsujimoto and Shimizu, 2000). All Bcl-2 family members possess at least one of four conserved motifs known as Bcl-2 homology domains (BH1–BH4). Most anti-apoptotic members, including Bcl-2 and Bcl-x_L, contain all the four domains. Pro-apoptotic members such as Bax and Bak lack the BH4 domain, whereas other pro-apoptotic members, the so-called BH3 domain-only proteins that include Bid, Bim and Bad, contain only the BH3 domain (Gross *et al*, 1999; Tsujimoto and Shimizu, 2000).

Recently, it has been demonstrated that Bax plays an essential role in inducing apoptosis in response to stress stimuli, as revealed by gene disruption of Bax and of Bax and Bak (Knudson *et al*, 1995; Lindsten *et al*, 2000; Wei *et al*, 2001; Zong *et al*, 2001). Bax is localized mostly in the cytoplasm, but redistributes to mitochondria in response to stress stimuli (Hsu *et al*, 1997; Wolter *et al*, 1997). After translocation to mitochondria, Bax induces cytochrome *c* release either by forming a pore by oligomerization in the outer mitochondrial membrane, or by opening other channels (Shimizu *et al*, 1999; Saito *et al*, 2000; Kuwana *et al*, 2002). The mechanisms underlying Bax translocation, however, are not fully understood.

Recent studies have shown that several proteins including 14-3-3 proteins prevent apoptosis through sequestration of Bax (Samuel *et al*, 2001; Guo *et al*, 2003; Nomura *et al*, 2003; Sawada *et al*, 2003). 14-3-3 proteins, which include seven isoforms in humans, are also implicated in antagonizing apoptotic signals through association with other pro-apoptotic proteins such as Bad, FKHRL1, ASK1 and Nur77 (Zha *et al*, 1996; Datta *et al*, 1997; Brunet *et al*, 1999; Zhang *et al*, 1999; Masuyama *et al*, 2001; van Hemert *et al*, 2001; Yaffe, 2002). Most 14-3-3 target proteins require phosphorylation to interact with 14-3-3, and consensus phosphoserine containing 14-3-3 binding motifs (RSXpSXP and RXXXpSXP) have been defined (Muslin *et al*, 1996; Yaffe *et al*, 1997). On the other hand, there are several examples of proteins containing dramatic variations from these motifs, including some that do not even require phosphorylation for binding, such as exoenzyme S, the platelet glycoprotein IB-IX-V complex, the plasma membrane H^+-ATPase AHA2 and *Drosophila* PAR-1 (Du *et al*, 1996; Masters *et al*, 1999; Svennelid *et al*, 1999; Benton *et al*, 2002). Bax also interacts with 14-3-3 proteins in a phosphorylation-independent manner (Nomura *et al*, 2003). A substantial proportion of Bax molecules is bound to 14-3-3 proteins in the cytosol of healthy cells; in response to stress stimuli, however, Bax dissociates from 14-3-3 and redistributes to mitochondria (Nomura *et al*, 2003). Moreover, caspases activated by stress stimuli cleave 14-3-3θ within its COOH-terminal region and promote its dissociation from Bax (Nomura *et al*, 2003). These observations are consistent with previous results showing that disruption of the 14-3-3σ gene promotes Bax translocation to mitochondria in response to cellular stresses (Samuel *et al*, 2001). However,

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the translocation of Bax to mitochondria occurs independently of caspase activation in a number of systems (Putcha *et al*, 1999; Gilmore *et al*, 2000; Tsuruta *et al*, 2002), suggesting the existence of another mechanism responsible for the dissociation of Bax from 14-3-3 proteins.

The c-Jun N-terminal kinase (JNK) represents a group of mitogen-activated protein kinases (MAPKs), which is activated when cells are exposed to environmental stresses (Davis, 2000). In all, 10 members of the JNK family are generated by the alternative splicing of transcripts derived from the JNK1, JNK2 and JNK3 genes. The studies of JNK gene disruption in mice have confirmed that JNK contributes to stress responses (Davis, 2000). JNK3 is essential for apoptosis of hippocampal neurons following exposure to excitotoxic stresses (Yang *et al*, 1997). JNK1 and JNK2 are required for apoptosis of thymocytes in response to ligation of the T-cell receptor and of neurons in the developing hindbrain (Kuan *et al*, 1999; Sabapathy *et al*, 2001). Furthermore, JNK1 and JNK2 double-knockout cells are resistant to apoptosis induced by UV, anisomycin or DNA damage (Tournier *et al*, 2000). Moreover, recent studies have shown that Bax and Bak are required for JNK-induced apoptosis, and that Bax remains inactive upon exposure of JNK-deficient fibroblasts to environmental stress (Lei *et al*, 2002). How then does JNK activate Bax?

In this study, we show that 14-3-3ζ and 14-3-3σ are direct targets of JNK and that phosphorylation of 14-3-3 proteins by JNK results in dissociation of Bax from 14-3-3 proteins, leading to apoptosis. This novel function of JNK may provide the missing link between the stress-activated kinase cascade and Bax translocation to mitochondria, a critical step in the regulation of apoptosis.

Results

Active JNK promotes Bax translocation to the mitochondria

We first examined whether JNK activation is sufficient to promote Bax translocation to the mitochondria in COS-1 cells, since Bax has been reported to be required for JNK-induced apoptosis in mouse embryonic fibroblasts (Lei *et al*, 2002). To this end, we utilized a green fluorescent protein (GFP)-Bax fusion construct to monitor Bax localization in real time (Tsuruta *et al*, 2002). In all the experiments with GFP–Bax, p35, a pan-caspase inhibitor, was co-transfected to block caspase activation induced by overexpression of GFP–Bax. As a constitutively active form of JNK, we used a fusion protein (MKK7–JNK) in which MKK7α1 and JNK1β1 are connected by the Flag epitope tag (Figure 1A); in this construct, which is similar to the one used by Lei *et al* (2002), MKK7 phosphorylates and activates JNK1 intramolecularly (K Yoshioka, unpublished data). We found that expression of MKK7–JNK (wild type, WT) promoted GFP–Bax translocation to the mitochondria (Figure 1B and C). In contrast, the fusion construct of MKK7α1 and a kinase negative JNK (MKK7–JNK (KN)) had no effect on GFP–Bax localization (Figure 1B and C), suggesting that JNK promotes Bax translocation by phosphorylation of some target(s). To determine if JNK activity triggers translocation of the endogenous Bax protein, the distribution of endogenous Bax was assessed by subcellular fractionation after transfection of COS-1 cells with the MKK7–JNK constructs. The amount of endogenous Bax detected in the mitochondrial fraction was increased, and that detected in the cytosolic fraction was decreased, by expression of MKK7–JNK(WT) but not by that of MKK7–JNK(KN). On the other hand, the abundance of the mitochondrial marker F_0F_1-ATPase subunit α (or that of the cytosolic marker α-tubulin) in the corresponding fraction was unaffected by the expression of either construct (Figure 1D). Although expression of MKK7–JNK(WT) resulted in the activation of caspase-3 in COS-1 cells (Supplementary Figure 1) (Lei *et al*, 2002), coexpression of p35, which prevented caspase-3 activation by MKK7–JNK(WT) (Supplementary Figure 1), failed to inhibit Bax translocation to the mitochondrial fraction (Figure 1D), suggesting that MKK7–JNK(WT) promoted the translocation of endogenous Bax to mitochondria independently of caspase activation.

To determine whether JNK activity is required for stress-induced translocation of Bax to the mitochondria, we examined the effect of SP600125, a JNK inhibitor, on GFP–Bax redistribution induced by the protein synthesis inhibitor anisomycin. Inhibition of protein synthesis by anisomycin triggers apoptosis in a broad variety of cells. Treatment of COS-1 cells with anisomycin resulted in phosphorylation of the JNK substrate c-Jun, and pretreatment with SP600125 blocked this effect of anisomycin (Figure 2A). Anisomycin also induced a gradual redistribution of GFP–Bax to mitochondria, and this effect of anisomycin was inhibited by SP600125 (Figure 2B). We also measured Bax translocation to the mitochondria using subcellular fractionation and found that SP600125 inhibited the translocation of endogenous Bax to mitochondria in response to treatment with anisomycin (Supplementary Figure 2). The concentration of SP600125 required for the inhibition of Bax translocation was the same as that required for the inhibition of c-Jun phosphorylation (data not shown). To verify that the effect of SP600125 was specific for JNK, we also used the JNK-binding domain peptide (JBD) (Dickens *et al*, 1997) and a dominant-negative (DN) JNK to block JNK activity. The JBD blocked anisomycin-induced GFP–Bax translocation (Figure 2C) and expression of DN form of JNK blocked GFP–Bax translocation induced by staurosporine (Supplementary Figure 3), another agent known to induce apoptosis. Taken together, these results suggest that JNK activity is required for Bax translocation to the mitochondria induced by anisomycin or by staurosporine.

JNK can regulate Bax localization independently of c-Jun, Akt and Bim

We first tested the ability of JNK to phosphorylate Bax and found that Bax is not phosphorylated by JNK in an *in vitro* kinase assay (Supplementary Figure 4). We therefore hypothesized that there is another JNK target that regulates Bax localization in response to stress stimuli. Apoptosis induced by neurotrophic factor deprivation in sympathetic neurons is mediated by the JNK-catalyzed phosphorylation of c-Jun (Harris and Johnson, 2001; Putcha *et al*, 2001; Whitfield *et al*, 2001). We examined whether c-Jun phosphorylation is required for Bax translocation to the mitochondria. MKK7–JNK(WT) phosphorylated c-Jun and induced activation of c-Jun-dependent transcription, as measured by a luciferase reporter gene controlled by an AP-1-dependent promoter (which monitors the activity of the Jun–Fos complex).

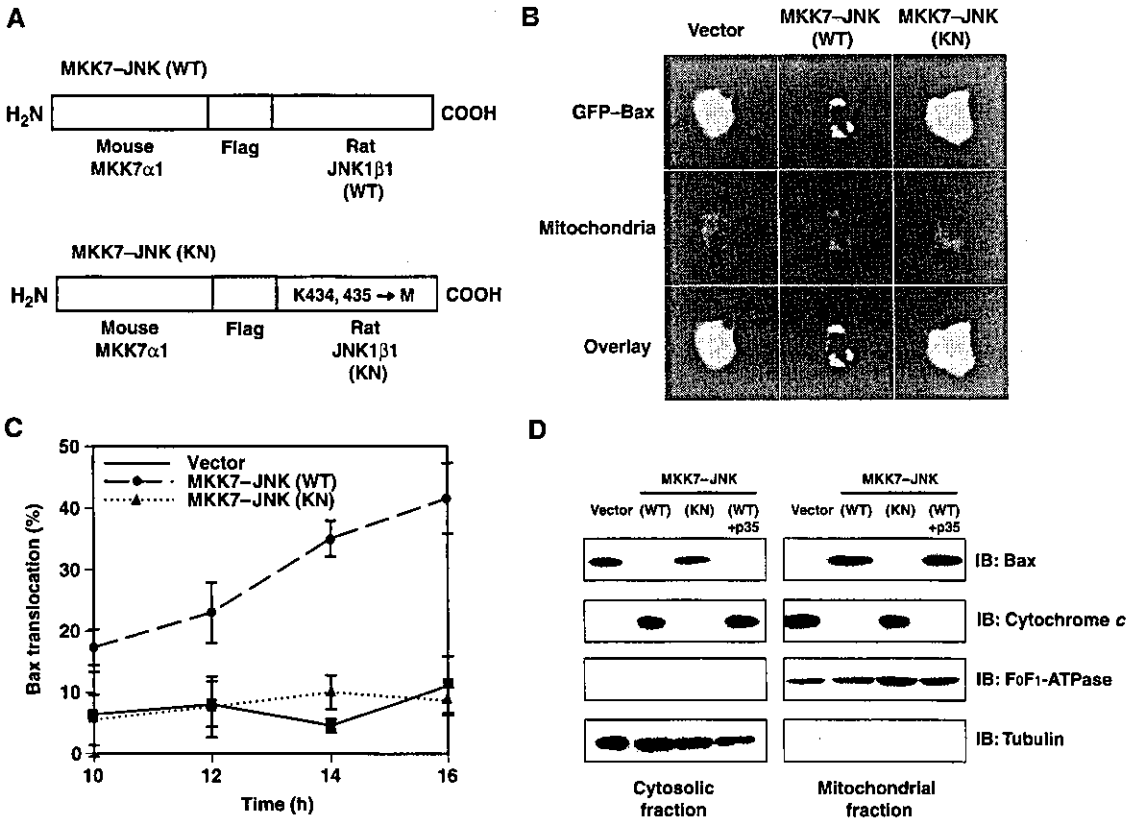

Figure 1 Expression of active JNK promotes Bax translocation to the mitochondria. (**A**) Schematic representation of the structure of the MKK7–JNK fusion proteins (WT or KN), which comprise mouse MKK7α1 linked to rat JNK1β1 by the Flag epitope sequence. The kinase-negative mutant of JNK1 was generated by replacement of Lys^{434} and Lys^{435} with methionines. (**B**) COS-1 cells were transfected for 13 h with expression vectors for GFP–Bax, the mitochondrial marker DsRed-Mito and the caspase inhibitor p35, together with a vector for MKK7-JNK (WT or KN) or the corresponding empty vector, as indicated. They were then examined for the distribution of GFP–Bax (green) and mitochondria (red) by fluorescence microscopy; the two separate images for each representative cell are also shown superimposed (overlay). (**C**) COS-1 cells were transfected for the indicated times as in (B), and the percentage of cells exhibiting GFP–Bax localization to mitochondria was determined. Data are means ± s.d. of values obtained from five fields of 30–150 cells in each of three independent experiments. (**D**) COS-1 cells were transfected for 20 h with expression vectors for GFP and MKK7–JNK(WT or KN) in the absence or presence of a vector for p35, as indicated. The transfection efficiency was ~75%, as determined by monitoring the expression of GFP. The cells were then subjected to subcellular fractionation, and the amount of endogenous Bax and that of cytochrome *c* in the mitochondrial and cytosolic fraction were assessed by immunoblot analysis (IB) with antibodies specific for these proteins. The amounts of the mitochondrial marker F_0F_1-ATPase and cytosolic marker α-tubulin were similarly assessed as an internal standard.

Activation of c-Jun-dependent transcription was blocked by coexpression of a DN form of c-Jun (Figure 3A). However, expression of DN c-Jun did not inhibit the translocation of GFP–Bax to mitochondria induced by MKK7–JNK(WT) (Figure 3B). The MKK7–JNK(WT)-induced increase in the amount of endogenous Bax in the mitochondrial fraction was also unaffected by DN c-Jun (Supplementary Figure 5). These results therefore suggest that JNK induces Bax translocation to mitochondria independently of the transcriptional activity of c-Jun.

Since previous reports have shown that Bax translocation to the mitochondria in response to apoptotic stimuli is suppressed by the PI3K–Akt pathway (Yamaguchi and Wang, 2001; Tsuruta *et al*, 2002; Molton *et al*, 2003), we examined the involvement of the PI3K–Akt pathway in JNK-mediated Bax translocation. We found that expression of MKK7–JNK(WT) or of MKK7–JNK(KN) had no effect on the EGF-induced activation and phosphorylation of Akt at Ser-473 in COS-1 cells (Supplementary Figure 6). Moreover, expression of constitutively active or DN Akt constructs did not affect the level of JNK phosphorylation (Tsuruta *et al*, 2002) or the JNK-induced GFP–Bax translocation to the mitochondria (Supplementary Figure 7). These results suggest that JNK does not induce Bax translocation by inhibiting the PI3K–Akt pathway.

The Bcl-2 family member Bim can regulate Bax translocation and has been reported to be a target of JNK (Lei and Davis, 2003; Putcha *et al*, 2003). We therefore examined whether Bim is phosphorylated by JNK under the conditions used in this study and whether Bim phosphorylation correlates with Bax translocation to mitochondria. Bim phosphorylation was monitored by a shift in its electrophoretical mobility on SDS–PAGE as described (Lei and Davis, 2003; Putcha *et al*, 2003). Immunoblot analysis showed a mobility shift of Bim in response to anisomycin treatment. The JNK inhibitor SP600125, however, had little effect on the anisomycin-induced mobility shift of Bim, whereas it effectively blocked phosphorylation of c-Jun (Supplementary Figure 8). Importantly, expression of MKK7–JNK(WT) promoted Bax translocation to mitochondria, but did not induce the mobility shift of Bim (Supplementary Figure 8). These data suggest that a kinase or kinases other than JNK are activated by

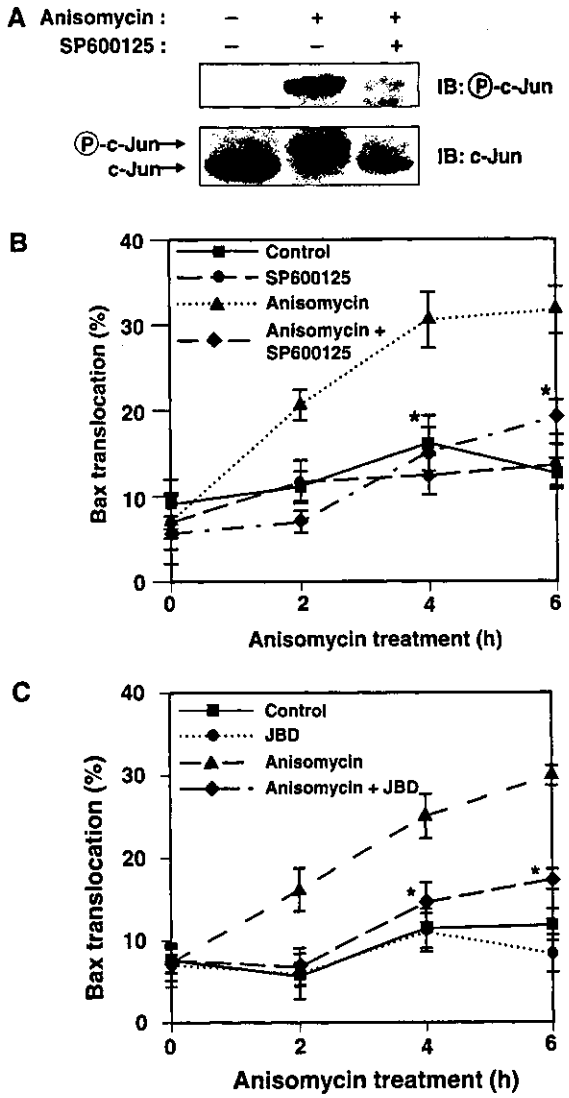

Figure 2 JNK is required for stress-induced translocation of Bax to the mitochondria. (**A**) COS-1 cells were incubated first for 30 min with or without 20 μM SP600125 and then for 1 h in the presence or absence of anisomycin (10 μg/ml). Cell lysates were then subjected to immunoblot analysis with antibodies to phosphorylated c-Jun or to c-Jun. The anisomycin-induced shift in the electrophoretic mobility of the band detected by the antibody to c-Jun reflects phosphorylation of c-Jun. (**B**) COS-1 cells were transfected for 11 h with expression vectors for GFP–Bax and p35, were pretreated with or without 20 μM SP600125 for 30 min, and were then incubated for the indicated times in the presence or absence of anisomycin (10 μg/ml). The percentage of cells in which GFP–Bax was localized to mitochondria was then determined. Data are means ± s.d. of values obtained from five fields of 30–150 cells in each of three independent experiments (*$P<0.0005$ as compared with the anisomycin group). (**C**) COS-1 cells were transfected for 11 h with expression vectors for GFP–Bax and p35, together with a vector for JBD or the corresponding empty vector, as indicated. The cells were then incubated for the indicated times in the presence or absence of 10 μg/ml anisomycin, after which the percentage of cells exhibiting GFP–Bax localization to mitochondria was determined. Data are means ± s.d. of values obtained from five fields of 30–150 cells in each of two independent experiments (*$P<0.0005$ as compared with the anisomycin group).

anisomycin and phosphorylate Bim, and that JNK induces Bax translocation independently of Bim phosphorylation at least in this system.

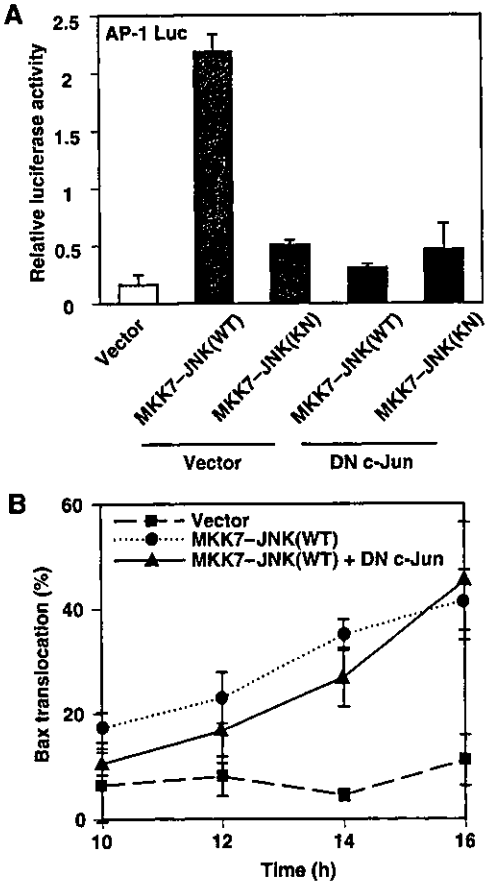

Figure 3 JNK induces Bax translocation to the mitochondria independently of c-Jun. (**A**) COS-1 cells were transfected for 1 day with an AP-1-luciferase reporter plasmid and expression vectors for MKK7–JNK(WT or KN) and a DN form of c-Jun or the corresponding empty vectors, as indicated. The normalized luciferase activity of cell lysates was then determined. Data represent the means ± s.d. of triplicate determinations from three independent experiments. (**B**) COS-1 cells were transfected for the indicated times with expression vectors for GFP–Bax and p35 together with vectors for MKK7–JNK(WT or KN) and DN c-Jun, as indicated. The percentage of cells exhibiting GFP–Bax localization to mitochondria was then determined. Data are means ± s.d. of values obtained from five fields of 30–150 cells in each of two independent experiments.

JNK phosphorylates 14-3-3ζ at Ser-184 and 14-3-3σ at Ser-186 in vitro

Under resting conditions, 14-3-3 proteins function as cytoplasmic anchors of Bax and prevent Bax from translocating to the mitochondria (Nomura *et al*, 2003). Consistent with this observation, targeted disruption of the 14-3-3σ gene was shown to accelerate Bax translocation to the mitochondria in human HCT116 cells (Samuel *et al*, 2001). These findings prompted us to investigate the possibility that JNK promotes Bax translocation to mitochondria by regulating the interaction between Bax and 14-3-3 proteins. Previous reports have shown that several isoforms of 14-3-3 are phosphorylated *in vivo* at a serine residue in the region between α-helices 7 and 8 (Ser-186 of 14-3-3β and Ser-184 of 14-3-3ζ), although the kinase or kinases responsible for this phosphorylation were not identified (Aitken *et al*, 1995). Interestingly, this residue conforms to the consensus sequence for JNK phosphorylation (Ser-Pro), and resides within the putative Bax-binding region (Figure 4A) (Nomura *et al*, 2003). We thus