Cell cycle changes after denervation of the newt limb regenerate, studied in vitro

M. Oudkhir, B. Boilly and B. Lassalle

Laboratoire de Morphogenèse animale, UA 685, Université des Sciences et Techniques de Lille, F-59655 Villeneuve d'Ascq Cedex (France), 19 December 1984

Summary. The proliferation of the mesenchyme of medium bud stage blastemas of *Pleurodeles,* measured by ^{3}H-thymidine incorporation and mitotic index, decreases about 40–50% under denervated conditions when cultivated in vitro for 4 days; epidermis is not affected in this case. Autoradiography of blastemas after ^{3}H-thymidine long term labeling shows that ¾ of the mesenchymal cells and ¼ of the epidermal cells are cycling when the blastema is innervated; there is no significant change of these percentages when the blastema is denervated. The results show, contrary to in vivo experiments, that denervation does not provoke an exiting from the cell-cycle but only lengthening of the cycle of the mesenchymal cells (probably of the G_1 phase).
Key words. Regeneration; denervation; cell-cycle; organ culture; amphibian; *Pleurodeles.*

We have previously shown that, in *Pleurodeles waltlii* Michah, in vivo denervation of a regenerating limb (at medium bud stage) is followed by a significant decrease in cell proliferation. The decrease is explained by a lengthening of the G_1 phase[1] and by the promotion of an exiting from the cell cycle[2]. To investigate the mechanisms controlling cellular proliferation in the blastema, we have developed an in vitro system[3]. In these in vitro conditions, it was important to check the effects of nervous tissue on the blastemal proliferation. Here we report the behavior of regenerating blastema cells under in vitro conditions in the presence or absence of nervous tissue (spinal ganglion).

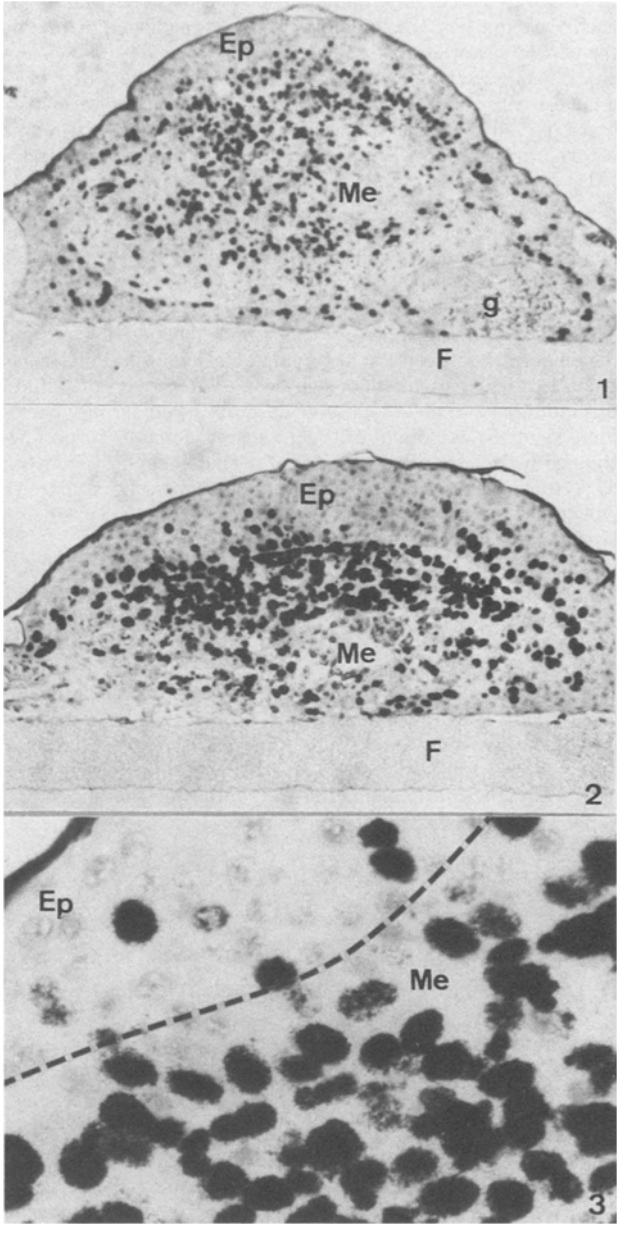
Methods. Adult *Pleurodeles waltlii* Michah (about 1 year old) were amputated bilaterally under MS 222 (Sandoz) anesthesia (1:1000, w/v) through the stylopod, near the elbow and allowed to regenerate at 23 ± 1 °C. When the blastemas reached the medium bud stage (about 15 days later) they were removed and sterilized as previously described[3]. They were cultured at 25 °C on supporting grids in Leibovitz medium (70% of L15 Gibco) to which antibiotics (penicillin, 100 U/ml, streptomycin, 100 U/ml) and 0.035 U/ml bovine insulin (Choay, Paris) were added. The blastemas were cultivated either without ganglion (denervated situation) or in association with a dorsal root ganglion taken from the spinal nerves 3 and 4 or 16 and 17 (innervated situation).

Three different measurements of proliferation were used:

1) ^{3}H thymidine incorporation measured by scintillation counting. Blastemal DNA synthesis was measured in both the innervated and denervated situations after 3–4 days of culture, the final 24 h of which were with 3 µCi/ml of ^{3}H-thymidine (sp. act. 1 Ci/mM, CEA) present in the culture medium. The radioactivity incorporated in large molecules of the epidermis and mesenchyme were measured separately on a Wallac 1215 LKB scintillation counter by the protocol previously described[1]. The incorporation of ^{3}H-thymidine is expressed as a function of the quantity of DNA which was determined by the spectrofluorimetric technique of Brunk et al.[4].

2) Mitotic index. Mitotic indices were determined on 7-µm sections stained with the Feulgen Rossenbeck reaction. Mitoses (prophase to telophase) which are randomly distributed in the entire blastema and the total number of cells were scored in every second section of the regenerate. The total number of cells was scored. A minimum of 10,000 cells were counted for each blastema. The mitotic index was then calculated by finding the number of cells in mitosis as a percentage of the total number of cells.

3) Autoradiography of blastemas after ^{3}H-thymidine long term labeling. In order to distinguish the cycling cells from non-cycling cells (i.e. cells which do not incorporate ^{3}H-thymidine), we cultured the blastemas in the presence of ^{3}H-thymidine over a prolonged period. Since the cell cycle lasts about 48 h, in previous work[2] we labeled for 48 h and 96 h to determine the quantity of cells which were cycling. We have shown that the number of cycling cells identified after 48 h is not much less than that after 96 h. Because of culture conditions in this study we were limited to 48 h of continuous labeling between the second



Figures 1–3. Autoradiographs of medium bud stage blastema cultured for 4 days (^{3}H-thymidine was present during the last 2 days of culture). About ¾ of mesenchymal cells (Me) and about ¼ of epidermal cells (Ep) are labeled. Fig. 1. Blastema co-cultured with a ganglion (g); ×135. Fig. 2. Blastema cultured alone; ×135. Fig. 3. Enlargement of 2; ×1080.

Percentages (±SD) of ^{3}H-thymidine incorporation (Inc. ^{3}H), the mitotic index (MI) and the labeling index (LI) of the epidermis (Ep) and the mesenchyme (Me) from denervated blastemas with respect to the controls (blastemas with ganglia) for different culture times (T). An asterisk indicates that the difference between the denervated blastemas and the controls is significant ($p < 0.05$). In parentheses: number of cases

	T	Ep	Me
Inc. ^{3}H	72 h	(10) 102 ± 35	(10) 47* ± 40
	96 h	(9) 74 ± 33	(9) 48* ± 32
MI	96 h	(3) 136 ± 31	(4) 62* ± 21
LI	96 h	(3) 96 ± 31	(3) 89 ± 27

and fourth days of culture. Following the labeling period the blastemas were prepared for autoradiography. The total number of cells was scored. A minimum of 5000 cells were counted for each blastema. The labeling index was then calculated by finding the percentage of marked cells.

Results and discussion. Cell proliferation in denervated blastemas is expressed as a percentage of that in innervated blastemas for all three of the measurements (table): ^{3}H-thymidine incorporation (Inc ^{3}H), mitotic index (MI) and labeling index (LI).

The results show that after the 3rd day, denervation causes a decrease of ^{3}H-thymidine incorporation close to 50% for the mesenchyme. Similarly, the mitotic index of the mesenchyme drops 40% with denervation. Neither of these parameters has significantly changed in the epidermis.

The difference in labeling indices for both the mesenchyme and the epidermis between the denervated blastemas and blastemas associated with ganglia is not significant. After 4 days of culture, this index is close to 24% in both cases in the epidermis. Therefore, ¾ of the epidermal cells can be considered to be non-cycling. However, the labeling index of the mesenchyme is between 67% (denervated situation) and 75% (innervated situation). Only ¼ of the mesenchymal cells can, therefore, be considered non-cycling (figs. 1, 2, 3).

After 4 days of culture, in vitro denervation essentially affects the proliferation of mesenchymal cells. They show a decrease of 40% (mitotic index) to 50% (^{3}H-thymidine incorporation). The proliferation rate of the epidermis is not significantly influenced by the denervation under these conditions. However, the effects caused by the denervation procedure are weaker in vitro than in vivo. In vivo 4 days after denervation[1] there is a 70–75% diminution of mesenchymal cell proliferation, and the proliferation of the epidermal cells falls 50–60%. It is likely that the presence of a significant amount of insulin (0.035 U/ml) somewhat masks the effects of denervation in vitro, and explains the discrepancy between the results obtained in vivo and in vitro.

Nevertheless, the results obtained in vitro concerning the influence of denervation on the proliferation of mesenchymal cells and the relative independence of the epidermis agree with those observed in vivo[1,5].

We have shown[6] by cytophotometry that, in vivo, the diminution of proliferation which follows denervation results from an accumulation of blastemal cells in a cell cycle phase with a DNA content equal to the 2C amount. This behavior is due to both a lengthening of the G1 phase and an exiting from the cell cycle[2]. This anticipated exiting from the cell cycle is not provoked by denervation in vitro, as is shown by the nearly identical labeling indices for blastemas cultured with and without ganglia. The labeling index for the mesenchyme (67–75%) is about three times the one measured in the epidermis (24%). This difference in the percentage of cycling cells in the two tissues may account for the weaker sensitivity of the epidermis to denervation as seen in vivo[1].

Even though denervation in vitro does not provoke an exiting from the cell cycle by the regenerating cells, it is clear that the observed fall of the proliferation indices indicates a lengthening of the cycle time for mesenchymal cells. It remains to be determined whether the G1 phase is the affected phase in vitro as it is in vivo.

In vivo denervation causes lengthening of the G1 phase which then terminates in exiting from the cycle into G_{0-1} [review in Rothstein[7]].

It seems that in vitro only the first part of this phenomenon, the lengthening of G1, takes place without the cells exiting from the cycle. Perhaps the culture conditions (addition of a growth factor like insulin) and/or the short duration of the culture (4 days) do not permit the cells to reach an exiting from the cell cycle as seen after denervation under in vivo conditions.

1 Boilly, B., Oudkhir, M., and Lassalle, B., Dev. Growth Differ. *27* (1985) 129.
2 Boilly, B., Oudkhir, M., and Lassalle, B., Biol. Cell., in press.
3 Lassalle, B., Biol. Cell *47* (1983) 187.
4 Brunk, C.F., Jones, K.C., and James, T.W., Analyt. Biochem. *92* (1979) 497.
5 Geraudie, J., and Singer, M., J. exp. Zool. *203* (1978) 455.
6 Oudkhir, M., Boilly, B., Lheureux, E., and Lassalle, B., Differentiation *29* (1985) 116.
7 Rothstein, H., Int. Rev. Cytol. *78* (1982) 127.

0014-4754/86/020189-02$1.50 + 0.20/0

H-Y antigen expression in heterogametic males (XY) and females (ZW): a factor in reproductive strategy?

T.J. Yang

Department of Pathobiology, University of Connecticut, Storrs (Connecticut 06268, USA), 26 February 1985

Summary. The cells of heterogametic females with ZW sex chromosomes express H-Y or H-W antigen. A hypothesis is formulated to explain why these animals are capable of 'practicing' *amphigonia retardata*, i.e., delay in actual fertilization of eggs by retaining viable sperm within the oviduct for a considerable time (several months).

Key words. H-Y antigen; H-W antigen; heterogametic females (ZW); amphigonia retardata.

Cells from males but not females of all mammalian species tested, including human, have been shown, directly or indirectly, to express H-Y or a cross-reactive cell surface antigen[1,2]. Apparently H-Y antigen[3] is evolutionarily highly conserved and the sex-specific antigen is found in the cells of the heterogametic sex of all vertebrate species[1–4]. It has been proposed that H-Y antigen directs the initially indifferent embryonic gonad to become a testis in male heterogametic species such as the mouse and an ovary in female heterogametic species such as the chicken[5–7]. In chickens, the female-specific histocompatibility locus coded antigen, H-W, which cross-reacts with H-Y is present on cells from female members of the species[8–10]. Female cells from other