Early Lesion of N-Nitroso-N-ethylurea-induced Hamster Neurofibromatosis Model

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ABSTRACT

To investigate histogenesis of neurofibroma, early lesions of N-nitroso-N-ethylurea-induced neurofibroma of Syrian golden hamsters were examined. The lesions were detectable from 8 weeks of age, and a total of 14 lesions in 9 hamsters from 8 to 12 weeks of age were observed. Most of the lesions were found in the skin; they were also observed in the trigeminal nerve, cervical plexus, and abdominal sympathetic ganglion. Histological examination revealed there were two types of early lesions such as solitary and plexiform types. The former developed in the s.c. or dermal part of the skin and showed invasive growth of the surrounding tissue, while the latter originated from the large nerves such as the trigeminal nerve or cervical plexus. Growth kinetics of the early lesions was quantitated by continuous administration of bromodeoxyuridine and double immunostaining. Using these systems we observed that various kinds of cells had already participated in early lesions and later Schwann cells became a main component of the tumor. The histogenesis of neurofibroma was considered to be complexed by proliferation of several kinds of cell type at the early stage.

INTRODUCTION

Neurofibroma is a slowly proliferating benign tumor of the peripheral nervous system (1). The tumor is also a central lesion for NF1, in which some of neurofibromas are congenital and the tumors progressively increase in number and size (2). The main component of the neurofibroma is considered to be the Schwann cell; however, unlike schwannoma, the tumor also contains heterogeneous components such as the perineurial cell, fibroblastic cell, and mast cell (1, 3). Since there are difficulties in examining the early developed lesion of neurofibroma in human cases, it has not been solved when the cellular heterogeneity occurs and what types of the cells are the true neoplastic components.

We induced multiple neurofibromas, melanomas, Wilms' tumors, and pheochromocytomas by transplacental administration of ENU to Syrian golden hamsters and considered this tumor-bearing hamster as an animal model for human NF1 (4). Like human neurofibroma, ENU-induced hamster neurofibroma showed nodular and infiltrative growth in the dermis or subcutis, plexiform growth, and differentiation to Meissner's corpuscle. Since in this model dermal or s.c. neurofibromas are detectable from 6 months of age, we can detect the early lesions when we examine the skin of younger animals. The purposes of the study reported here were to investigate how neurofibroma grows at the early stage and to learn the nature of the proliferative component in the lesion. To address these questions we examined early lesions of neurofibromas in this model, growth kinetics of the tumor using continuous administration of BrdUrd, followed by double immunostaining with anti-BrdUrd and anti-S-100 protein and considered the histogenesis of neurofibroma.

MATERIALS AND METHODS

Animals and Induction of the Early Lesion of Neurofibroma. Transplacental induction of hamster neurofibroma was performed as described previously (4). Briefly, pregnant Syrian golden hamsters (purchased from Clean Experimental Animal Center, Co., Sugito-machi, Saitama, Japan) received a single i.p. injection of 100 mg/kg of ENU (Nakarai Chemical Co., Kyoto, Japan) on the 16th day of gestation.

The offspring were weaned at 28 days of age, separated by sex, and fed with basal diet MHF (Oriental Yeast, Inc., Tokyo, Japan) with tap water ad libitum. Three female offspring were sacrificed and subjected to autopsies in the every 4, 8, 10, and 12 weeks of age.

Administration of BrdUrd. Alzet osmotic minipumps (model 2001; Alza Corporation, Palo Alto, CA) were implanted s.c. into the hamsters and 1 mg of BrdUrd (Takeda Yakuin Co., Osaka, Japan) per h was administered for 6 days before the sacrifice.

After the skin of the whole body was stripped off and cut into 5-mm thicknesses, paraffin-embedded thin sections were made together with the sections of trigeminal nerves, cervical and lumbar plexus, and abdominal sympathetic ganglia. Early lesions of neurofibroma were examined on routine hematoxylin and eosin-stained slides by light microscopy. When the s.c. and i.d. lesions were detected, they were further investigated for cell kinetics study by immunostaining together with all the trigeminal nerves and cervical and lumbar plexus sections. Multiple sections of the skin, trigeminal nerves, and cervical/lumbar plexus from the age-matched female offspring of untreated mothers were examined as the same manner. The 33-week-old female offspring in which the fully developed s.c. neurofibromas were recognized also received the administration of BrdUrd for 6 days and subjected to autopsies; then, the tumors were removed for paraffin sections and double immunostaining.

Cell Kinetics Study by Double Immunostaining. Double immunostaining for BrdUrd and S-100 protein was carried out using combined PAP (5) and biotin-streptavidin alkaline phosphatase (6) methods. Paraffin-embedded sections were laid on poly-L-lysine-coated slides, deparaffinized, and digested by 0.1% trypsin (Sigma Chemical Co., St. Louis, MO) for 5 min at 37°C. After denaturation by 0.1 N hydrochloric acid for 20 min at 37°C, the slides were covered by the mixture of mouse monoclonal anti-BrdUrd (Becton Dickinson, Mountain View, CA) and rabbit polyclonal anti-S-100a (DAKO, Santa Barbara, CA) overnight at 4°C. Then, the slides were treated sequentially by the mixture of swine anti-rabbit IgG (DAKO) and biotinylated goat anti-mouse IgG (Biogenex, Dublin, CA), the mixture of rabbit IgG-PAP complex (DAKO) and alkaline phosphatase-conjugated streptavidin (Biogenex).

After alkaline phosphatase-labeled anti-BrdUrd products were detected by fast blue BB salt (Sigma), PAP-labeled anti-S-100a binding sites were detected by aminoethyl carbosol (DAKO).

The number of BrdUrd- and S-100-positive cells in early lesions and...
fully developed tumors was determined, and the rates of BrdUrd(+)/all cells, BrdUrd(+)-S-100(+) and BrdUrd(+)-S-100(+) were calculated in 1000 cells when the lesion contained enough cells. Statistical analysis was performed using Student's \( t \) test.

**RESULTS**

**Occurrence and Localization of the Early Lesions.** The occurrence and localization of the neurofibroma early lesions were summarized in Table 1. The early lesion was defined as a neoplastic proliferation less than 3 mm in diameter. The earliest lesion was detectable in the 8th week of age; then, they increased in number up to 7 lesions per 3 hamsters in the 12th week. The most common site of the early lesion was the s.c. part of the skin followed by the trigeminal nerve.

**Histological Findings of the Early Lesions.** The i.d. and s.c. lesions did not show a significant relation with the thick nerve fascicles and showed invasive growth of the surrounding tissue. The hair follicles, sweat glands, and thin nerve fibers were frequently involved in the lesions (Fig. 1a). These lesions were considered to be the early lesions of solitary neurofibroma. A considerable number of the mast cells were recognized in the lesions (Fig. 1b), indicating that these lesions consisted of not a single cell population. A very small early lesion at the 8th week consisting of only a small number of the cells around thin nerve fibers was observed (Fig. 1c).

The hypercellular areas, which caused thickening of the nerve fascicles, were observed in the trigeminal nerve and cervical plexus (Fig. 1d). Proliferating within the nerve fascicles and remaining covered by perineurium, these lesions were considered to be the early lesions of plexiform neurofibroma. In this type of the lesion there was no mast cell infiltration when the lesions were ensheathed by perineurium, and if the lesions had grown out from the perineurium the mast cells had been observed in the lesions.

**Double Immunostaining.** Because of slowly proliferative characteristics of the hamster neurofibroma continuous administration of BrdUrd for long period was performed using osmotic minipumps. BrdUrd incorporation increased significantly, however, BrdUrd-positive nonneoplastic cells such as leukocytes, macrophages, vascular endothelial cells, etc., were also increased. The double immunostaining using anti-BrdUrd monoclonal and anti-S-100 polyclonal antibodies was therefore carried out in order to exclude these nonneoplastic cells and to detect Schwann cell lineage of the early lesions.

The staining showed blue BrdUrd(+) nuclei and red S-100(+) cytoplasms of Schwann cell lineage (Fig. 2a–c). A small lesion detected on the hematoxylin and eosin-stained slide showed that there were BrdUrd(+) cells around the thin nerve fiber and some of BrdUrd(+) cells were also positive for anti-S-100 (Fig. 2a). These findings confirmed that the lesion was indeed an early lesion of neurofibroma containing Schwann cell lineage.

The early lesions of the trigeminal nerve and cervical plexus showed significant increase of the BrdUrd-positive cells in the nerve fascicles (Fig. 2b). On the other hand, there was no BrdUrd(+) cell in the nerve fascicles obtained from the age-matched control animals by continuous 6-day labeling.

The number of BrdUrd(+) cells was counted and total labeling indexes were calculated for the early lesions and fully developed neurofibromas (Table 2). The labeling index of the early lesions was slightly higher than that of the fully developed neurofibromas; however, the difference was not statistically significant. There were a considerable number of BrdUrd(+) and S-100(−) cells in the early lesion of hamster neurofibroma (Fig. 2c). The number of the populations of BrdUrd(+) and S-100(−) were also counted, and the labeling index of the Schwann cell and the rate of the Schwann cell among the proliferating cell were calculated. Table 2 also showed the rate of BrdUrd(+) and S-100(+) cells, which indicated the BrdUrd labeling index of Schwann cell lineage. The average rates were 6.7% in the early lesions and 4.0% in the fully developed neurofibromas by 6-day labeling (the difference, not significant).

The rate of BrdUrd(+) and S-100(+) indicated the rate of the Schwann cell among all the kinds of the proliferating cell. The average rate of the early lesions was 21.3%, whereas the well-developed neurofibromas obtained from 33-week-old hamsters showed the increased Schwann cell number with the average rate of 38.7%. The difference of the average rates between both the groups was statistically significant \( (P<0.01) \). In the early stage the larger lesion tended to show the higher rate. In fully developed neurofibroma BrdUrd(+) cells tended to lie towards the periphery of the tumor masses or to form small aggregates. In contrast, early lesions showed diffuse localization of BrdUrd(+) cells.

**DISCUSSION**

There were morphologically two types in the early lesions of hamster neurofibroma. The first type, a dermal or s.c. lesion, was considered to be originated from the very peripheral part of the nerve fibers. It showed infiltrative growth and formed a nodule, believed to be an early lesion for solitary type neurofibroma. The other type was an early lesion for plexiform type neurofibroma which occurred in the more central site of the nerve such as the trigeminal nerve and cervical plexus than the former type.

BrdUrd is a thymidine analogue incorporated into DNA and is useful for examining the cell kinetics of the various neoplastic and nonneoplastic cells (7). On routine examination of the labeling index of the tumors BrdUrd is administered by one shot injection, and after 30–90 min the tissue is fixed and subjected for the immunocytochemical procedure. Both human and experimental hamster neurofibromas are slowly proliferating tumors, and in hamster tumors the routine one shot administration of BrdUrd yielded only a little labeling index less than 0.5% (data not shown). Since continuous labeling of BrdUrd using osmotic minipumps enabled improved detection of the proliferating cells (8), we utilized the method and confirmed that an extensively increased labeling index was available.

However, the number of apparently nonneoplastic BrdUrd(+) cells in the early lesions as well as definite neurofibromas was increased significantly by this method, and this phenomenon led to increased difficulty in understanding the role of Schwann cell proliferation on the early growth of neurofibroma and the

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**Table 1 Distribution of the early lesions of neurofibroma**

<table>
<thead>
<tr>
<th>Age* (wk)</th>
<th>Skin</th>
<th>Tri.N.</th>
<th>C.Pix.</th>
<th>L.Pix.</th>
<th>Ab.Gg.</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>12</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>7</td>
</tr>
</tbody>
</table>

*All age groups consisted of 3 animals each.

*Tri.N., trigeminal nerve; C.Pix., cervical plexus; L.Pix., lumbar plexus; Ab.Gg., abdominal sympathetic ganglion.
exact nature of the tumor. In order to characterize the component of proliferating cells in neurofibroma and its early lesion we tried to perform double labeling for BrdUrd and S-100 protein, which is the marker for schwannian differentiation (9) and is not expressed in perineurial cells and fibroblasts. The double immunostaining, which enabled extensive analysis of the relationship between cell proliferation and differentiation (10), was a powerful tool to evaluate the kind of proliferating cells in the heterogeneous cellular components. Besides, using a specific marker such as S-100, contamination of nonneoplastic cells can be excluded in the cell cycle study (11). The proliferating activity of total cell component in the early lesions was slightly higher than in the fully developed neurofibromas; however, the Schwann cell component in the proliferating cell group was rather small in number in the former, and it increased significantly in the latter. The finding suggested that in the early stage of neurofibroma the various kinds of the cells such as the Schwann cell, fibroblastic cell, perineurial cell, mast cell, etc., proliferated in the tumor; however, in the later stage of Schwann cell shared the main part of tumor proliferation.

The cellular heterogeneity of neurofibroma has been emphasized by several investigators (3, 12, 13). In our study participation of the various kinds of cells in the early stage and frequent involvement of the neighboring tissue such as sweat glands and hair follicles were considered to be important for heterogeneous components and morphological varieties of neurofibroma.

It may be very important to investigate if neurofibroma is indeed “heterogeneous.” The frequent occurrence of S-100(-) BrdUrd(+) cells in the early lesion suggested several possibilities as follows.

**Mechanism 1.** The true neoplastic component is only the Schwann cell, which releases some growth-promoting factors to induce migration and proliferation of fibroblasts, perineurial cells, and mast cells. Although the exact growth-promoting effect of the Schwann cell for other cell lineages has not been fully understood to date, it is known that the close cell-cell interaction between several cell types plays an important role on formation and regeneration of the peripheral nervous system (14, 15). Moreover, the results of the present study that the early lesions of plexiform type unsheathed by perineurium did not contain any mast cells and that the mast cells infiltrated as soon as the lesions invaded out from the perineurium strongly suggested that the Schwann cell induces the mast cell migration.

**Mechanism 2.** The early lesions contain primitive and immature cells of Schwann cell lineage which do not express immunohistologically detectable levels of S-100 protein. In this case our double immunostaining may not detect these immature schwannian lineage. It may be effective to perform double labeling for BrdUrd and other markers of Schwann cell lineage for detection of more primitive cells.

**Mechanism 3.** Not a single cellular component is neoplastic, namely there are neoplastic Schwann cells and fibroblastic and/or perineurial cells. Whereas Schwann cells are developed from...
 EARLY LESION OF NEUROFIBROMA

Table 2: Rate of BrDUrd(+/all cells, BrDUrd(+) S-100(+/S-100(+), and BrDUrd(+) S-100(+/BrdUrd(+) in the early lesions and fully developed neurofibromas.

<table>
<thead>
<tr>
<th>Age/site</th>
<th>BrDUrd(+/all cells (%)</th>
<th>BrDUrd(+) S-100(+/S-100(+)</th>
<th>BrDUrd(+) S-100(+/BrdUrd(+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early lesions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10w:Ab.Gg.</td>
<td>16.5</td>
<td>5.7</td>
<td>9.2</td>
</tr>
<tr>
<td>10w:skin</td>
<td>13.6</td>
<td>11.2</td>
<td>14.5</td>
</tr>
<tr>
<td>12w:skin</td>
<td>6.0</td>
<td>7.6</td>
<td>26.6</td>
</tr>
<tr>
<td>12w:skin</td>
<td>7.3</td>
<td>5.2</td>
<td>39.7</td>
</tr>
<tr>
<td>12w:skin</td>
<td>6.4</td>
<td>6.6</td>
<td>22.6</td>
</tr>
<tr>
<td>12w:C.Pix.</td>
<td>7.4</td>
<td>4.0</td>
<td>15.2</td>
</tr>
<tr>
<td>Mean</td>
<td>9.5*</td>
<td>6.7*</td>
<td>21.3*</td>
</tr>
<tr>
<td>Fully developed neurofibromas</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>33w:skin</td>
<td>6.1</td>
<td>2.9</td>
<td>37.7</td>
</tr>
<tr>
<td>33w:skin</td>
<td>11.2</td>
<td>7.0</td>
<td>42.2</td>
</tr>
<tr>
<td>33w:skin</td>
<td>4.4</td>
<td>2.0</td>
<td>45.6</td>
</tr>
<tr>
<td>33w:skin</td>
<td>8.6</td>
<td>6.4</td>
<td>40.7</td>
</tr>
<tr>
<td>33w:skin</td>
<td>4.2</td>
<td>2.3</td>
<td>31.6</td>
</tr>
<tr>
<td>33w:skin</td>
<td>6.5</td>
<td>3.0</td>
<td>34.6</td>
</tr>
<tr>
<td>Mean</td>
<td>6.8*</td>
<td>4.0*</td>
<td>38.7*</td>
</tr>
</tbody>
</table>

* w. weeks; Ab.Gg., abdominal sympathetic ganglion; C.Pix., cervical plexus.

No significant difference between the mean value of early neoplastic lesions and fully developed neurofibromas.

Statistically significant (P < 0.01) between the mean value of early neoplastic lesions and fully developed neurofibromas.

be required for formation of neurofibroma (20, 21). Further analysis of the nature and function of the NF1 gene product will clarify the pathogenesis of neurofibroma; however, it is believed that the various factors are necessary for the progression of the tumor. Study is in progress to examine if there are any abnormalities of the NF1 gene in our hamster neurofibroma model.

In summary the present study revealed cellular heterogeneity of neurofibroma even in its early lesion. Schwann cells, however, increased and became the main component as the tumor developed. Further study is needed for our understanding of the histogenesis of neurofibroma, cell-cell interaction of the peripheral nervous system, and differentiation of the Schwann cell.

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REFERENCES


Fig. 2. a, immunohistochemistry of the early lesion at the 8th week using double labeling for BrDUrd and S-100. Blue BrDUrd(+) and red S-100(+) cells around the small nerve fiber. × 290. b, immunohistochemistry of early lesion of the trigeminal nerve. BrDUrd(+) nuclei in the lower half. × 260. c, BrDUrd(+) nuclei and S-100(+) cytoplasms in individual cells. Note considerable numbers of BrDUrd(+) S-100(−) and BrDUrd(−) S-100(+) cells. × 400.

the neural crest (16), the origins of endoneurial fibroblasts and perineurial cells have not been clarified, and they have distinct characteristics compared with the Schwann cell (17), suggesting that Mechanism 3 is not feasible. In contrast, Mechanism 1 would be appealing in the view of the behavior of the mast cell. Besides, Mechanism 2 is also possible and the two possible mechanisms might be cooperative in the formation of hamster neurofibroma.

Recently the NF1 gene has been mapped to chromosome 17q and its sequence has been determined in part (18–20). Loss or mutation of at least one allele of the NF1 gene is considered to

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