Modifications of Tumor Histology by Point Mutations in the v-fps Oncogene: Possible Role of Extracellular Matrix

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ABSTRACT

Fujinami sarcoma virus (FSV) encodes a protein-tyrosine kinase, p130\(^{\text{FSV}}\), whose enzymatic activity and ability to transform cultured cells to a neoplastic phenotype are reduced by substitution of the major autophosphorylation site tyrosine-1073 with other amino acids. We compared the histopathology of tumors formed in syngeneic immunocompetent rats by Rat-2 cells and by Rat-2 cells transformed in culture with (a) wild type (wt) FSV, (b) mutant FSV where the codon for tyrosine-1073 of p130\(^{\text{FSV}}\) had been changed to codons for phenylalanine or serine, and (c) a revertant FSV, genotypically identical to wt FSV, in which the codon for tyrosine-1073 had been restored. Latency periods from cell inoculation to tumor formation were 12-29 weeks with Rat-2 cells, 6-8 weeks with mutant-transformed Rat-2 cells, and 2-4 weeks with wt FSV- and revertant FSV-transformed Rat-2 cells. Untransformed Rat-2 cells formed tumors that histologically resembled low grade fibrosarcomas or fibromas and were characterized by uniform fusiform cells in parallel arrays with a prominent collagenous stroma. The growth pattern of tumors produced by mutant FSV-transformed cells was generally similar, although cellular forms and intercellular organization were less uniform. In contrast, Rat-2 cells transformed with either wt FSV or revertant FSV produced tumors that resembled highly malignant sarcomas and were composed of diffuse sheets of pleomorphic, disorganized cells and stroma rich in hyaluronate but poor in fibrous components. Local invasion occurred in 25% of tumors produced by Rat-2 cells and in 53 and 36% of tumors formed by mutant FSV- and wt FSV-transformed cells, respectively. In culture, Rat-2 cells and mutant FSV-transformed cells produced fibrillar pericellular matrices of collagen I and fibronectin. From 5 to 15% of protein secreted by these cells was collagen. Cultures of wt FSV- and revertant FSV-transformed cells lacked collagen and fibronectin matrices and collagen secretion was reduced to 0-2%. These results show that clinically relevant histological characteristics of malignant tumors can correlate with single amino acid substitutions previously shown to affect the enzymatic activity and transforming ability of an oncogenic protein tyrosine kinase. The mechanisms underlying some of the histological differences in this system may be related to differences in the production of extracellular matrix components among the transformed cells.

INTRODUCTION

Histopathological criteria are used routinely in cancer diagnosis and treatment planning. However, the molecular basis for the variations in cellular characteristics, intercellular organization, and growth patterns, which underlie these criteria, is largely unknown. This is particularly true for poorly differentiated malignant neoplasms (1-4).

In the present study, we examined the effects on tumor

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5 The abbreviations used are: FSV, Fujinami sarcoma virus; wt, wild type; FN, fibronectin; PBS, phosphate-buffered saline; p 130, M, 130,000 protein (other proteins are similarly designated).
MATERIALS AND METHODS

Cell Culture and Animal Inoculation. The development of the FSV-transformed rat cell lines used in this study has been described in detail elsewhere (9, 10). Briefly, Rat-2 cells (12) were transfected with either wt FSV DNA or FSV DNA which had been specifically mutated using oligonucleotide-directed mutagenesis. Cells were grown at 37°C in 5% CO2-air in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Monolayers of subconfluent cells were harvested by trypsinization, washed twice in Tris-saline plus 10% fetal bovine serum and twice with Tris-saline, and finally resuspended in Tris-saline for injection into animals. For each experiment, syngeneic, immunocompetent female Fischer 344 rats 4 to 6 weeks old, in groups of three to four, were given s.c. injections in the loose skin at the back of the neck of approximately 1 x 106 cells in 0.1 ml/animal. Five separate experiments were carried out over a 2-year period. Rats were examined every 1 to 2 weeks for the appearance of palpable tumors at the site of injection. The latency period is expressed as the time required postinjection for the appearance of palpable tumors.

Histology, Histochemistry, and Immunocytochemistry. For general histopathological examination, the tumors were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin-eosin. The growth pattern within many tumors varied, particularly in areas of degeneration. To reduce this source of variability, comparisons between tumors were based only on regions of healthy, growing tumor tissue, which were selected by the presence of mitotic figures and by maximal distance from areas of necrosis. Paraffin-embedded sections were stained with hyaluronate with Alcian blue, (pH 2.5), with testicular hyaluronidase treatment (BDH Chemicals) and staining at pH 1.0 as controls. For the demonstration of collagen, sections were stained with Masson's trichrome. FN was demonstrated immunocytochemically. Sections of ethanol-fixed tissue were deparaffinized and stained with avidin-biotin-peroxidase according to the method of Hsu et al. (14), using goat antiserum to rat FN (Calbiochem) as the first antibody. Multiple sections from each case were graded and classified blindly by two independent investigators (N. A. and A. W.). The animals were not examined for metastases.

Immunofluorescence Microscopy of Cultured Cells. Cells were grown on glass coverslips for 1 to 6 days, to variable confluence. To demonstrate FN, the coverslips were rinsed and incubated for 1 h at 37°C with serum-free medium and then fixed in cold (—20°C) methanol. They were permeabilized with acetone, air dried, rehydrated in PBS, and stained with goat anti-rat FN (Miles Laboratories, Elkhart, IN) for 1 h, followed by rhodamine-labeled swine anti-goat IgG (Tago, Inc., Burlingame, CA) for 30 min. The stained coverslips were rinsed and soaked for 2 h in PBS and then mounted in Gelvatol (15), pH 7.8. They were photographed with Zeiss epifluorescence optics on Tri-X England Nuclear) in 1.0 ml medium. The cellular/pericellular matrix was examined for FN (Fig. 4). Rat-2 cells produced a conspicuous fibrillar

RESULTS

Histopathology

Although growth patterns varied within individual tumors, a single pattern usually predominated. The majority of tumors formed by any one of the different cell lines had distinct characteristics (Figs. 1 and 2; Table 1). Untransformed Rat-2 cells formed tumors after 12—29 weeks. These tumors were composed of long interlacing bundles of small fusiform cells with inconspicuous intercellular spaces, interspersed with a fibrous stroma. Local muscle invasion was observed in 25% of tumors, after latency periods of 19 weeks or more. These tumors were classified as fibromas or low grade fibrosarcomas.

Rat-2 cells transformed with the mutants FSV-F(1073) or FSV-S(1073) induced tumors after latency periods ranging from 6 to 8 weeks. Histologically, the cells in these tumors varied more in size and orientation although most were again fusiform with inconspicuous intercellular spaces, and were aligned in parallel arrays along connective tissue trabeculae. Most of these tumors, too, were classified histologically as low grade fibrosarcomas and had many features in common with the tumors produced by untransformed cells. No distinction could be made between tumors produced by line FSV-F(1073) and tumors produced by line FSV-S(1073). Tumors produced by cells transformed with wt FSV or the revertant FSV-Y(1073) appeared after latency periods of 2—4 weeks. They resembled one another but differed from tumors formed by Rat-2 cells and mutant-transformed Rat-2 cells. Histologically, they were grade IV, highly malignant pleomorphic sarcomas. Although these cells formed bundles in some areas, the tumors were largely composed of diffuse, disorganized cell sheets. The cells were pleomorphic, round, or polygonal; often vacuolated; and interspersed with mononucleated and multinucleated giant cells. Intercellular spaces were prominent while fibrous stroma was inconspicuous. Mutant-transformed and wt FSV- or FSV-Y(1073)-transformed cells invaded adjacent host tissues in 53 and 36% of tumors, respectively (Table 1; Fig. 2). Neovascular channels formed in all Rat-2 tumors, in 53% of FSV-F(1073) or FSV-S(1073) tumors, and in none of the wt FSV or revertant FSV. No vascular invasion was observed in any of the tumors. The animals were not examined for distant metastases.

Masson's trichrome stain demonstrated a collagenous stroma in Rat-2, FSV-F(1073), and FSV-S(1073) tumors, which was most prominent in regions of reduced mitotic activity. In addition to a fine fibrillar framework present in all 3 groups of tumors, those tumors formed by mutant-FSV-transformed cells were characterized by prominent collagenous trabeculae. In contrast, collagen was sparse in tumors formed by wt FSV- and revertant FSV-transformed cells (Fig. 3). Hylarionate, as demonstrated by Alcian blue staining, was most conspicuous in intercellular spaces of tumors formed by wt FSV- and revertant FSV-transformed cells. Fibronectin was present in all tumors with no consistent quantitative difference among groups (Fig. 3).

Extracellular Matrix Production in Culture: Immunofluorescence Microscopy

FN (Fig. 4). Rat-2 cells produced a conspicuous fibrillar pericellular FN matrix, which appeared after seeding and increased with time and cell density. It was located on the basal and apical cell surfaces. The FN production in cultures of the FSV-S(1073) line was comparable to that of Rat-2 cells in every respect. Cells of the FSV-F(1073) line also secreted FN but in smaller amounts and in less fibrillar form than Rat-2 cells. Neither wt FSV-transformed nor revertant FSV-transformed cells deposited any pericellular FN matrix, although there was cytoplasmic fluorescence suggestive of FN synthesis.

Collagen (Fig. 5). In dense cultures of Rat-2 cells, a fine network of pericellular fluorescent material was demonstrated...
Fig. 1. A, tumor produced by Rat-2 cells. Bundles of uniform, small, spindle-shaped cells in parallel arrays. Many normal mitotic figures. Consistent with fibroma or low grade fibrosarcoma. B, tumor produced by mutant FSV-F(1073)-transformed Rat-2 cells. Similar to A although more atypical as indicated by increased variability in cell size and less distinct alignment into bundles. C, tumor produced by wt FSV-transformed Rat-2 cells, resembling highly malignant fibrosarcoma. Sheets of randomly oriented, pleomorphic cells with distinct intercellular spaces, interspersed with mono- and multinucleated giant cells. D, tumor produced by revertant FSV-Y(1073)-transformed Rat-2 cells. The cellular characteristics and intercellular organization resemble those in C. H & E, × 300.
with antibody to collagen I. Similar fluorescence occurred in cultures of mutant-transformed cells but was negligible in cultures of wt FSV- or revertant FSV-transformed cells.

Collagen Content of the Cellular-Pericellular Matrix and Culture Medium

Five independent assays gave similar results. In Rat-2 cultures, 5–7% of cellular-pericellular matrix protein and up to 30% of all secreted protein was procollagen. Procollagen synthesis and secretion were absent or greatly reduced in wt FSV-transformed and revertant FSV-transformed cells. However, in both mutant lines, procollagen synthesis levels resembled those of Rat-2 cells. A representative experiment is shown in Table 2.

DISCUSSION

In experimental systems, tumor histology is not generally used as an indicator of oncogenicity since there are other, more direct means available to evaluate the malignant potential of cell populations. However, in clinical tumor pathology, histological criteria have for a long time formed the basis for diagnostic and prognostic decisions, and the histological grading of tumors continues to play an essential role in cancer management. This reliance on grading is based on extensive evidence for correlations between the histological appearance and the malignant potential of tumor cell populations in human cancer. The histopathological criteria used routinely to make diagnostic and prognostic decisions describe, in the final analysis, the properties and biological behavior in vivo of malignant cell populations. However, little is known about the specific factors which determine the phenotype and the clinical grade of malignant neoplasms.

Recent reports have suggested that gene amplification, chromosome translocation, or point mutation might activate cellular oncogenes during the formation of human tumors (5). Changes in culture morphology, transforming potential, and biochemical activity due to single amino acid substitutions in transforming proteins such as p130, p21ras, and p185 have been reported (9, 10, 18, 19). In addition, cells transformed by a mutant Rous sarcoma virus in which tyrosine-416 of p60v-src was replaced with phenylalanine have reduced tumorigenicity in immunocompetent animals (20). The present study demonstrates that the substitution of a single amino acid in an oncogene-encoded protein can, in turn, elicit clinically relevant changes in tumor histology.

### Table 1: Predominant histological characteristics of the tumors (hematoxylin-eosin)

Data are based on multiple sections of each tumor, classified blindly by two independent investigators (N. A. and A. W.). Evaluations were limited to areas of healthy tumor tissue, which were selected by the presence of mitotic figures and by maximal distance from areas of necrosis.

<table>
<thead>
<tr>
<th>Cell type (No. of tumors)</th>
<th>Growth pattern</th>
<th>Connective tissue trabeculae</th>
<th>Muscle invasion</th>
<th>NC, clefts</th>
<th>Cell size</th>
<th>Cell shape</th>
<th>Wide intercellular spaces</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diffuse</td>
<td>Bundles</td>
<td>No. %</td>
<td>No. %</td>
<td>No. %</td>
<td>No. %</td>
<td>No. %</td>
</tr>
<tr>
<td>Rat-2 (12)</td>
<td>0</td>
<td>12</td>
<td>100a</td>
<td>9</td>
<td>75</td>
<td>3</td>
<td>25</td>
</tr>
<tr>
<td>S(1073) (5)</td>
<td>0</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>0</td>
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<tr>
<td>F(1073) (10)</td>
<td>2</td>
<td>8</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Total mutant (15)</td>
<td>2</td>
<td>13</td>
<td>86</td>
<td>10</td>
<td>66</td>
<td>8</td>
<td>53</td>
</tr>
<tr>
<td>wt FSV (7)</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Y(1073) (4)</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Total wt (11)</td>
<td>10</td>
<td>90</td>
<td>1</td>
<td>10</td>
<td>2</td>
<td>18</td>
<td>4</td>
</tr>
</tbody>
</table>

* NC, neovascular channels; GC, mono- and multinucleated giant cells; Rat-2, untransfected; other cell types are Rat-2 transfected with: S(1073), serine mutant; F(1073), phenylalanine mutant; wt FSV, wild type FSV; Y(1073), revertant to wild type FSV.

* Percentage values greater than 50% are in italics.
Fig. 3. Extracellular matrix composition of tumors. A–D, collagen, Masson's trichrome stain, × 300. A, untransformed Rat-2 cells. Intercellular collagen network (arrowheads). B, FSV-F(1073) tumor. Cells orient along collagen trabeculae (arrowheads). C, FSV-S(1073) tumor. Stellate growth pattern with extensive collagenous stroma. D, wt FSV tumor. Intercellular spaces appear empty. E, F, hyaluronate, Alcian blue, at pH 2.5 × 600. Hyaluronate (arrowheads) is sparse and limited to regions near collagenous trabeculae in the FSV-F(1073) tumor (E) but is prominent throughout the intercellular spaces of the wt FSV tumor (F). G, H, fibronectin, avidin-biotin-peroxidase, × 800. Nuclei are counterstained with hematoxylin. Fibronectin appears as a fibrous/granular deposit in the spaces between the nuclei of both the FSV-F(1073) tumor (G) and the wt FSV tumor (H) (stars). There was no consistent quantitative difference in fibronectin distribution among different tumor groups.
Fig. 4. Fibronectin (arrowheads) is deposited as a pericellular matrix in cultures of untransformed (A) and FSV-S(1073)-transformed (B) Rat-2 cells, but not in Rat-2 cultures transformed with wt FSV (C). Immunofluorescence microscopy, × 300.

Fig. 5. Collagen I is deposited as a pericellular matrix in cultures of untransformed (A) and FSV-F(1073)-transformed (B) Rat-2 cells, but not in Rat-2 cultures transformed with wt FSV (C). Immunofluorescence microscopy, × 300.

Table 2 Collagen synthesis by untransfected and transfected Rat-2 cells in culture (means ± SD)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cells and pericellular matrix</th>
<th>Medium</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat-2</td>
<td>5.7 ± 0.55</td>
<td>17.9 ± 6.82</td>
<td>9.9 ± 2.33</td>
</tr>
<tr>
<td>F(1073)*</td>
<td>9.9 ± 6.67</td>
<td>14.9 ± 4.18</td>
<td>11.7 ± 2.64</td>
</tr>
<tr>
<td>S(1073)*</td>
<td>4.5 ± 0.71</td>
<td>10.3 ± 3.06</td>
<td>6.3 ± 0.96</td>
</tr>
<tr>
<td>wt FSV*</td>
<td>1.8 ± 0.42</td>
<td>0.0 ± 1.45</td>
<td>1.0 ± 0.66</td>
</tr>
<tr>
<td>Y(1073)*</td>
<td>0.4 ± 1.17</td>
<td>0.2 ± 0.63</td>
<td>0.3 ± 0.54</td>
</tr>
</tbody>
</table>

* Based on [3H]proline incorporation of approximately 3 × 10⁶ cpm/culture. Quadruplicate cultures, 1.5–1.8 × 10⁶ cells/culture.

In our model system, v-fps oncogene action was not required to induce tumorigenicity, since untransformed Rat-2 cells produced tumors of low grade malignancy after long latency periods. Similar tumorigenicity of Rat-2 cells has been described by others, and some subclones have even been reported to metastasize in syngeneic animals after latency periods of up to 6 months (13).

Transfection with the mutant v-fps oncogene in our study caused only limited changes in the histology of the Rat-2 tumors. However, it did affect their biological behavior since the latency periods were reduced, and approximately 50% of the tumors invaded adjacent tissues within 8 weeks. A similar relationship exists between the biological behavior and histology of NIH/3T3 cells and NIH/3T3 cells transfected with an activated c-Ha-ras gene (21). In this study, NIH/3T3 cells were weakly tumorigenic in nude mice, while transfection with the c-Ha-ras gene accelerated tumor formation and greatly enhanced the metastatic potential of these cells. Yet, histologically, both transfected and untransfected cells formed fibrosarcomas that were indistinguishable from one another. Thus, in the NIH/3T3 system, as well as in ours, the enhanced invasiveness/metastatic potential does not correlate with major histological changes.

The latency periods required for tumor formation by cells transformed with wt FSV were significantly shorter than those of both mutant FSV-transformed and untransformed Rat-2 tumors. This step in tumor progression was associated with the appearance of histological characteristics of highly malignant sarcomas, namely loss of fibroblast-specific differentiation and intercellular organization, lack of neovascular channels, cellular pleomorphism, and replacement of a collagenous by a hyaluronate-rich extracellular matrix. Thus, the presence of the mutant v-fps oncogene was sufficient to reduce latency periods and increase invasiveness, but the cellular characteristics that conferred a higher histological grade of malignancy on the wt FSV tumors appeared to depend specifically on the presence of...
tyrosine-1073 in p130^{as-fps}. Similarly, in culture, the malignancy-related phenotypic characteristics of the mutant FSV-transformed lines are intermediate between those of Rat-2 cells and those of wt FSV-transformed cells (9, 10). It is important to note, however, that in the series of tumors studied here, short latency periods and highly abnormal histological features did not correlate with increased aggressiveness as indicated by invasiveness, since mutant FSV- and wt FSV-transformed cells invaded adjacent tissues equally efficiently.

The histochemical and immunocytochemical demonstration of differences in the stroma of the three groups of tumors suggested that changes in extracellular matrix may be responsible for some of the histological differences observed. There is much evidence indicating that an altered extracellular matrix composition of tumor stroma can arise as a result of altered synthesis by host cells in response to tumor cell influences (22-24). To determine whether or not such a phenomenon was responsible for the results observed here, extracellular matrix production was studied in culture, in the absence of host cells. The results suggest that altered matrix synthesized by the transformed cells contributed, at least in part, to the differences in stroma among the tumors.

In the Rat-2 cultures, transformation by wt FSV greatly reduced collagen production relative to total protein synthesis. This change parallels similar observations in a variety of avian and mammalian cells transformed with other oncogenic retroviruses (25-27). While the molecular basis for this phenomenon varies among cells and with collagen types, it appears to result from reduced transcription of the collagen genes in at least some cases (25, 27). Experiments with cell hybrids have provided strong evidence for a correlation between the ability to synthesize a collagenous extracellular matrix and suppression of malignancy in malignant cell/fibroblast crosses (28, 29). Suppression of malignancy in these hybrids was postulated to be the result of terminal differentiation with concomitant growth arrest. In the present report, the prominent collagen framework formed in the tumors of Rat-2 cells and mutant FSV-transformed cells, but not in tumors of wt FSV-transformed cells, was similarly an expression of fibroblast-specific terminal differentiation (16, 17). Thus, cessation of growth in differentiating subpopulations in these two groups of tumors may have contributed to their prolonged latency periods. It appears, therefore, that one of the transforming functions of the p130^{as-fps} protein is interference with fibroblast differentiation and that the tyrosine-1073 residue is required for this function.

In addition to effects on tumor progression, the well known influence of collagens on cell adhesion, shape, orientation, and directed movement (30-32) probably conferred upon the Rat-2 and mutant FSV tumors many of the cytological and histological characteristics common to fibromas and low grade fibrosarcomas. It is particularly interesting in this respect that in the cell hybrid studies (28), the collagen-producing, growth-inhibited tumors resembled histologically the mutant FSV tumors described here.

Transformation of cells with avian oncogenic retroviruses results in increased hyaluronate production (33). Two important biological effects of this glycosaminoglycan are the expansion of extracellular spaces and enhanced cell detachment (30). Hyaluronate also inhibits the differentiation of mesenchymally derived cells (34). It interacts in a highly specific fashion with cells directly (35) and also with other components of the extracellular matrix (36, 37). The increased amounts of hyaluronate in the stroma of tumors produced by the wt FSV-transformed cells in our study may have contributed to the expanded intercellular spaces and to the lack of fibroblast-specific intercellular organization and differentiation.

No consistent histological difference in FN content was observed among tumors formed by the different cell lines. In culture, however, both untransformed and mutant FSV-transformed Rat-2 cells produced a fibrillar pericellular matrix, while the revertant and wt FSV-transformed cells did not. These differences among the lines in culture are in keeping with many other studies where a pericellular FN matrix becomes reduced with progression to malignancy (38, 39). Such a transformation-induced change is frequently one in extracellular processing rather than in rates of synthesis and secretion, so that FN is released into the culture medium in a soluble form, rather than incorporated into a matrix. The similar amounts of FN observed in histological sections of all cell lines in the present study suggest that here, too, rates of FN secretion remained similar in the course of transformation. The microenvironment within tumors limits diffusion of macromolecules, and thus differences in pericellular FN that are observed in culture become obliterated in vivo. A similar explanation was proposed by Stenman and Vaheri (40), who found no histological difference in FN distribution between human sarcomas and benign soft tissue tumors, contrary to the situation in culture. Regardless of the lack of obvious quantitative differences in FN among the tumors, our observations in culture suggest that differences in the submicroscopic organization of FN may have contributed to the histological differences observed among the lines. It is of interest in this regard that the FN receptor is a substrate for tyrosine phosphorylation by p130^{as-fps}, suggesting that its interaction with FN may be directly modified by the v-fps oncoprotein (41).

Other cellular properties that distinguished the wt FSV tumors included cellular pleomorphism and the presence of giant cells. Since these characteristics depend on cytoskeletal functions and/or a particular distribution of cytoskeletal components, it is significant that wild type p130^{as-fps} may associate with the cytoskeletal matrix and that the distribution of actin filaments is altered in FSV-transformed cells with high levels of p130^{as-fps} (42-45). Perhaps some of the characteristics of the tumors depended directly on interactions of p130^{as-fps} with cytoskeletal elements, and these interactions were altered by substitution of tyrosine-1073.

Finally, the observations made in this study raise intriguing questions concerning the relative roles of the various matrix and cytoskeletal components in the changing phenotypic expression of the cell lines examined here. Further studies should provide information about possible reciprocal regulatory interactions among the genome, cytoskeleton, and extracellular matrix in this system (37) and about the mechanisms of transformation by the v-fps oncogene.

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