Characterization of neuronal differentiation factor(s) produced by Kagura-2 cells established from aflatoxin B₁-induced rat hepatoma

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ラット肝癌由来 Kagura-2 細胞が産生する神経分化誘導因子について

Summary

Recently we have demonstrated that the growth of Kagura-2 cells established from aflatoxin B₁ (AFB₁)-induced rat hepatoma was markedly stimulated by glucocorticoids (GCs). To investigate a possible role of GCs in the growth of Kagura-2 cells, we studied the effect of conditioned medium (CM) prepared from Kagura-2 cells on the neuronal differentiation of pheochromocytoma (PC12) cells.

Kagura-2 cell-CM caused in PC12 cells a number of physiological changes which mimic the nonreplicating sympathetic neuron-like cells, including induction of neurite extension and acetylcholinesterase activity. The neuronal differentiation factor(s) in CM were inactivated by the treatments of trypsin, acetic acid, dithiothreitol and heat. The molecular weights of factor(s) ranged from 6,000 to 14,000 on the basis of experiments using various molecular weight cut-off dialysis tubing and gel filtration.

AFB₁, a metabolite of *Aspergillus flavus*, is well known for its potency in hepatocarcinogenesis in experimental animals. AFB₁ as well as hepatitis virus has also been implicated in the etiology of human liver cancer, on the basis of statistically significant correlation between the increased occurrence of liver cancer and the consumption of grains contaminated with AFB₁ in Asia and Africa areas¹⁾.

We have demonstrated the over-expression of c-

myc and c-Ha-ras mRNA in AFB₁-induced rat hepatocellular carcinomas and cultured cell lines²⁾. Subsequently the activation of c-ras oncogene family by point mutation in AFB₁-induced rat liver cancers has been reported by McMahon et al.³⁾ and Sinha et al.⁴⁾ We have also demonstrated the modulation of hormonal induction of tyrosine aminotransferase and GC receptors by AFB₁ and sterigmatocystin in the rat hepatoma cell line⁵⁾. Most recently we have reported that the colony formation of Kagura-2 cells in soft agar was markedly enhanced by GCs, and the cells showed the GC-dependent growth in serum-free medium accompanying with the transient expression of c-myc gene^{6,7)}. Moreover Lu et al.⁸⁾ have demonstrated that the growth-stimulatory activity of various androgens or GCs on SC-3 cells established from mouse mammary tumor was mediated through a FGF-like peptide in an autocrine mechanism.

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We therefore studied the effect of Kagura-2 cell-CM on PC12 cells which differentiate into sympathetic neuron like cells in the presence of growth factors such as NGF, acidic FGF, basic FGF and IL-6^{9,10}. In this paper we describe the features of neuronal differentiation factor(s) produced by Kagura-2 cells.

Materials and Methods

Preparation of CM from Kagura-2 cells Kagura-2 cells were cultured in collagen-coated dishes containing Dulbecco's modification of Eagle's medium (DMEM) with 10% fetal calf serum (FCS) in a humidified CO₂ incubator at 37°C and the medium was changed every two other days. When the cells were grown up to 70–80% confluence, the medium was changed to serum-free medium and cultured for additional 2 days. This serum-free medium will be referred to as Kagura-2 cell-CM. The supernatant, after centrifugation at 5,000×g for 30 min, was filtered through a 0.22 µm nitrocellulose filter and used for experiments.

Neuronal differentiation of PC12 cells by CM

PC12 cells were plated at $2-3 \times 10^4$ cells per collagen-coated 35-mm dish and cultured in the growth medium consisted of DMEM with 10% FCS and 5% horse serum. One day after the plating, the growth medium was changed to the growth medium supplemented with Kagura-2 cell-CM (growth medium: CM=1:2, V/V) and cultured for 6 days with two changes of the medium.

Results and Discussion

Under routine culture conditions, PC12 cells proliferated as round chromaffin-like cells (Fig. 1-A). By the addition of NGF to the growth medium, PC12 cells flattened and began to send out short neurites within a few days, and after several days proliferation ceased and the majority of the cells formed a network of long and branched neurons (Fig. 1-B). As shown in Fig. 1-C, in Kagura-2 cell-CM supplemented growth medium, PC12 cells remained alive, and following a lag of 3 days, also started to extend neurites.

Fig. 1. Induction of the neuronal morphology of PC12 cells by NGF and Kagura-2 cell-CM. PC12 cells were maintained in growth medium (A), in growth medium supplemented with 50 ng/ml of mouse 2.5S NGF for 6 days (B) or growth medium supplemented Kagura-2 cell-CM as described in "Materials and Methods" for 6 days (C). The photographs were taken under phase-contrast optics. Magnification, $\times 200$.

To determine whether the changes induced by Kagura-2 cell-CM are involved in neuronal differentiation, we investigated whether neuron-specific properties known to be induced by NGF and cAMP are also induced by CM obtained from Kagura-2

Table 1. Acetylcholinesterase activities in PC12 cells treated with well known neuronal differentiation factors and Kagura-2 cell-CM

Treatment		Acetylcholinesterase activity (nmol/min/mg protein)	Ratio (treated/control)
Control			
In growth medium	(n=9)	17.8±0.49	1.00
NGF	(n=5)	37.3±1.84	2.10
Bt ₂ cAMP	(n=6)	39.9±2.19	2.24
basic FGF	(n=3)	17.9±1.18	1.01
Kagura-2 cell-CM generated in			
DMEM with 10% FCS	(n=7)	27.9±0.72	1.57
DMEM without FCS	(n=5)	34.0±1.75	1.91

PC12 cells (5×10^5 cells/35-mm dish) were treated with 50 ng/ml NGF, 1 mM Bt₂cAMP, 5 ng/ml basic FGF or Kagura-2 cell-CM for 6 days as described in "Materials and Methods." A colorimetric enzyme assays were performed with homogenates from 3 to 9 independent cultures for each group by the method of Ellman et al.¹¹⁾ Data are given as mean±s.e. n, number of experiments.

cells in PC12 cells. First, we observed that PC12 cells treated with CM contained higher activities of acetylcholinesterase (as did NGF- or cAMP-treated cells) than did untreated cells (Table 1). Second, Kagura-2 cell-CM induced the expression of c-fos gene within 30 min, although the level of induction was less than those by NGF and cAMP (data not shown).

The neuronal differentiation factor(s) were completely inactivated by the treatments of 100 µg/ml trypsin for 30 min at 37°C, those of 0.17 M acetic acid for 5 min at room temperature, those of 65 mM dithiothreitol for 2 hr at room temperature or heating for 5 min at 100°C. The molecular weights of the factor(s) ranged from 6,000 to 14,000 on the basis of experiments using various molecular weight cut-off dialysis tubing and Sephadex G-200 gel filtration.

These data show that Kagura-2 cells produce the neuronal differentiation factor(s) which are acid-unstable and relatively low molecular weight protein like as NGF. We are now investigating whether the factor(s) are essential for Kagura-2 cell growth as an autocrine growth factor.

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