論文の内容の要旨

論文題目　ＪＣウィルス誘発ハムスター髄芽腫由来のGFAP陽性細胞株

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ヒトのパポーバウィルスである JC ウィルスは実験動物に病理学的に多種にわたる脳腫瘍を発生させる。JC ウィルス Tokyo-1 株も同様に動物に高い確立で発癌性があることが報告されている。特に、ハムスターでは小脳髄芽腫を、ラットでは未分化脳腫瘍を発現させる。最近の報告では、この株はハムスターの小脳の外顆粒細胞層に高い親和性がありそれが高頻度に髄芽腫が発現する理由の一つであると考えられる。この Tokyo-1 株で誘発された髄芽腫を培養レベルで検索する過程において約２年目の継代培養細胞に GFAP (glial fibrillary acidic protein) 陽性の細胞が出現していることを見出した。もとの腫瘍細胞やその初代培養細胞では GFAP 免疫染色が陰性であったので、この GFAP 陽性細胞群は継代中に星細胞への分化形質を獲得した可能性が考えられる。この研究は培養細胞由来の GFAP 陽性細胞の樹立と性状分析について言及し、さらに髄芽腫の有する多様な方向への分化能力についてその一つの分化形質である GFAP についてとりあげて報告する。

材料と方法

細胞培養

用いた細胞は、ウィルスにより誘発されたハムスター髄芽腫由来の培養細胞（東京都神経研究所, 保井博士提供）である。培地は非必須アミノ酸添加のイーグル最小必要培地でウシ胎児血清（10%）およびゲンタマイシンを加えた。クローニングはマイクロプレートを用いた限界希釈法で行った。

免疫染色

カバーグラス上に培養した細胞を、冷 70% エタノールで１５分間固定後、免疫ペルオキシターゼ間接法、あるいは一部、PAP 法により免疫染色を行った。

用いた一次抗体は、モノクロン抗 GFAP（都精神研、芳賀博士）, 抗ビメンチン（Rockefeler 大学, Wang 博士）, 抗ニューロフィラメント構成蛋白（200K, 160K 及び 68K 都老人研, 井原博士）であり、その他、抗 GFAP, 抗S-100, 抗ニューロン特異的エノラーゼ（いずれも DAKO 社）, 抗グルタミンシンセターゼ（都神経研, 小田博士）, 抗 SV40T 抗原（東大医科研, 山口博士）である。

二次抗体は、HRP 標識抗ウサギ IgG ヤギ血清, HRP 標識抗マウス IgG ウサギ血清（いずれも Tago 社）を用いた。

免疫ブロット

培養細胞およびハムスター脳乳剤より 1% Triton X100, 0.6MKCL 不溶の粗細胞骨格分画を調整し、GFAP の有無を免疫ブロット法で検索した。

移植

$1X10^6$ 個の細胞をゴールデンハムスターの大腿部皮下又は、頭蓋内に注入した。成長した腫瘍はパラフィン包埋後、ヘマトキシリン染色と免疫染色を行った。又、腫瘍の一部を滅菌的に処理し、細胞を培養系に移した。

結果

用いた培養ハムスター髄芽腫細胞は GFAP 陰性で、継代維持されていたが、継代2年目の細胞について各種抗体を用いた免疫染色を行ったところ、約 60% の細胞が GFAP 陽性を示すことが判明した。そこでクローニングを行った結果、ほぼ 100% の細胞で GFAP 陽性を示すクローンが6個得られた。このうち、抗 GFAP 抗体の染色性がもっとも著しいクローンを I-23 細胞株と命名し、その細胞について検索した。
I-23 細胞株は、光顕では比較的多量の細胞質に富む三角形あるいは多角形の細胞が主で、まれに広く細胞質を張り出した大型細胞がみられる。I-23 細胞は抗 GFAP 抗体（モノクロンおよびポリクロン抗体）でほぼ 100% の細胞が強陽性に染色され、その染色像は網状，細線維状であった。この細胞は抗ビメンチン抗体でも強陽性に染色され、その染色像は抗 GFAP 染色の場合と同様、網状，細線維状であった。その他の蛋白（S-100, ニューロン特異的エノラーゼ, ニューロフィラメント構成蛋白 200K, 160K および 68K）に対する抗体では I-23 細胞は染色されなかった。
電顕による検索では、I-23 細胞の胞体に径10nm以下の細い線維の集塊が認められた。なお、クローン I-23 細胞は親細胞と同様、パポーウィルス共通 T 抗原がすべての細胞の核で陽性であった。

つぎに、抗 GFAP 抗体を用いた免疫ブロット法で I-23 細胞の粗細胞骨格分画を検索したところ、分子量 47K ダルトンの単一バンドが検出された。これはハムスター脳の細胞骨格分画が示す GFAP と同じ分子量であった。

I-23 細胞の移植実験では、１３匹全てのハムスターに３から９週の間に腫瘍が増殖した。腫瘍は硬くて、肉眼的に周囲の組織とは隔絶されていたが、組織学的にはヒトの線維性星細胞腫と類似していた。免疫組織学的検索では GFAP とビメンチン両方とも陽性で、その染色像は培養細胞と同様網状, 線維状であった。

さらに、この腫瘍細胞を培養系に移すと、培養細胞は I-23 細胞と同様の性質を有していた。以上の結果、JC ウィルスによる GFAP 陰性の培養細胞より GFAP 陽性の細胞株 (I-23) が確立された。

考察

I-23 細胞においては、抗 GFAP 抗体による免疫染色像が網状, 細線維状を示すこと、また Triton X100 不溶の粗細胞骨格分画に抗 GFAP 抗体陽性バンドが検出されることから、産生された GFAP が細線維の構造物として存在していると考えられる。

一般的に、培養細胞株はビメンチン陽性であることが多いが、I-23 細胞においてもビメンチンは陽性であり、その染色像は細線維状であった。I-23 細胞において GFAP とビメンチンは同一の線維を構成しているのかどうかに関しては今後の解析を要するところである。

脳腫瘍の初代培養ではGFAP やニューロフィラメント蛋白陽性の細胞を検出するのはまれではないが、これらの分化形質を示す細胞は比率としても少なく、また培養による脱分化という要素も加わって、継代を重ねるうちに消失してしまう場合が多い。今回の GFAP 陽性株樹立過程で興味深いのは、GFAP 陰性の原腫瘍（ JC ウィルス誘発

ハムスター髄芽腫）および培養早期の細胞からGFAP 陽性細胞の出現をみた点である。I-23 細胞は、パポーバウィルス共通 T 抗原と JC ウィルス（Tokyo-1 株）の DNA を有することから、この GFAP 陽性細胞株は継代途中で他から混入されたのではなく、ウィルスによりトランスフォームされた GFAP 陰性の髄芽腫より直接由来した細胞といえる。このもとの細胞より、GFAP がどのように発生したかを説明することは困難であるが、ヒトの髄芽腫細胞は、神経細胞，グリア細胞あるいは脳室上衣細胞などの方向へ分化する潜在能力を有することが示されている。これより JC ウィルス Tokyo-1 株によりトランスフォームされてできたハムスター髄芽腫のある細胞が星細胞へ分化する潜在能力を獲得したと思われる。この見解は JC ウィルス Tokyo-1 株が小脳外顆粒層の他方向への分化能を有する細胞へ感染しトランスフォームし、その結果、その細胞が内顆粒層へ移動して髄芽腫になるという最近の研究報告により支持されている。GFAP を有する I-23 細胞は確実に移植可能であり、その組織像はもとの髄芽腫とは著しく異なり、ヒトの線維性星細胞腫に酷似しており、又、GFAP も陽性であった。

従って、I-23 細胞は、常にGFAP を発現し、星細胞腫の組織像を呈することから、安定した星細胞の表現形態を有するように思われる。この細胞株はGFAP 産生のメカニズムばかりでなく、星細胞腫の生物学的な研究に有力な情報を提供すると考えられる。

A glial fibrillary acidic protein (GFAP) positive cell line derived from JC virus induced medulloblastoma in hamster

JCウィルス誘発ハムスター髄芽腫由来の glial fibrillary acidic protein (GFAP) 陽性細胞株

猪野屋　博

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(Summary)

A glial fibrillary acidic protein(GFAP) positive cell line was established from cultured cells of a GFAP negative hamster medulloblastoma that had been induced by the Tokyo-1 strain of JC papovavirus. The clone, referred to as I-23, was characterized by morphological, immunohistochemical and immunoblotting studies. I-23 cells were rich in cytoplasm and contained numerous fine fibrils. The cells were positive for GFAP and vimentin, with a reticular staining pattern, but negative for neurofilament triplet proteins, S-100 protein, neuron-specific enolase and glutamine synthetase. SV40 T antigen was present in all the cells. Immunoblotting studies of the crude cytoskeletal preparation from I-23 cells revealed a 47-kilodalton band, which was reactive with the antibodies to GFAP. Inoculation of I-23 cells into hamsters produced solid tumors with high efficiency. These tumors resembled to human astrocytoma in morphology and were positive for GFAP. It was easy to establish GFAP-positive cell lines from these tumors. The cultured cells again produced solid tumors in hamsters similar to the tumors produced by I-23 cells, with respect to astrocytoma-like histological patterns and the expression of GFAP. Thus, I-23 cells could be passaged either in vitro or through the combination of in vitro and in vivo, without losing the phenotypic expression of GFAP. It was considered that a portion of GFAP negative medulloblastoma cells, which might have the potential to undergo glial differentiation,

acquired the ability to produce GFAP through the in vitro passages.

Key words; Medulloblastoma, JC papovarirus, Glial fibrillary acidic protein(GFAP), Experimental brain tumor, Astrocytic differentiation.

Human papovavirus JC induces brain tumors with pathology of diverse range in experimental animals (Walker et al, 1973 and London et al, 1978 & 1984), since JC virus was isolated from brain tissue of a case of progressive multifocal leukoencephalopathy(Padgett BL et al, 1971). The Tokyo-1 strain of JC virus has also been demonstrated to be highly oncogenic in animals, frequently inducing cerebellar medulloblastoma in hamsters (Nagashima et al, 1984) and undifferentiated brain tumors in rats (Ohsumi et al, 1985). The high affinity of this strain to the cells in cerebellar external granular layer has recently been demonstrated in hamsters and was suggested to be one of the causative factors for frequent occurrence of medulloblastoma in this animal (Matsuda et al, 1987). During the course of our studies on the characterization of cultured cells from medulloblastoma induced by the Tokyo-1 strain, we noticed that a part of the cell population became positive for glial fibrillary acidic protein (GFAP) after blind passages for two years. Since the tumor cells in situ and the cultured cells in early passages had lacked immunoreactivity for GFAP (Nagashima et al, 1984), we considered the possibility that the positive cells might have acquired astrocytic differentiation during cultivation. The present study concerns the establishment and characterization of a GFAP positive clone from this culture and discusses the phenotypic expression of GFAP with respect to the multipotential nature of medulloblastoma cells (Rorke 1983).

Materials and methods

Cell culture

The original medulloblastoma cell line with a history of two-year passage was obtained from Dr. K. Yasui (Department of Bacteriology, Tokyo Metropolitan Institute for Neurosciences, Tokyo, Japan) and maintained at $37^{\circ}C$ in Eagle's minimum essential medium (MEM) containing non-essential amino acids, 10% fetal bovine serum (FBS) and Gentamycin. The cells received a weekly passage. Cloning was performed using a standard limiting dilution method on microplates.

Immunocytochemistry

Cells were culture on the cover slips. They were washed in phosphate-buffered saline (PBS) and fixed in a 70% ethanol solution at $0^{\circ}C$ for 15 min. Fixed cells were pretreated with 20% FBS in PBS for 30 min and incubated with primary antibodies and peroxidase-conjugated second antibodies at 37° C for 1 h, respectively. Between the incubations, the cells were washed with PBS. After the final washing, the reaction product was demonstrated with 0.04% diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide. Cells were counterstained with methylgreen. The primary antibodies used were as follows; monoclonal antibody to GFAP (provided by Dr. S. Haga, Psychiatric Institute of Tokyo, Tokyo,

Japan), rabbit antibody to GFAP (Dakopatts, Copenhagen, Denmark), rabbit antibody to glutamine synthetase (GS, provided by Dr. M. Oda, Tokyo Metropolitan Neurological Hospital, Tokyo, Japan), rabbit antibody to vimentin (provided by Dr. J. Wang, Rockefeller University, New York, USA), rabbit anti-neurofilament triplet proteins (200K, 160K, and 68K respectively; provided by Dr. Y. Ihara, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan), rabbit anti-S-100 protein (Dakopatts), and rabbit anti-neuron specific enolase(NSE, Dakopatts). For the demonstration of papovavirus T antigen, hamster serum containing antibody to SV40 T antigen (provided by Dr. Yamaguchi, Institute of Medical Sciences, University of Tokyo, Tokyo, Japan) was used. For second antibodies, peroxidase-conjugated goat antibody to rabbit IgG (Tago, Burlingame, USA) and peroxidase-conjugated rabbit antibody to mouse IgG (Tago) were used. The first antibodies were omitted for control studies of immunohistochemistry.

Electron microscopy

Cells were washed in cold PBS and fixed in phosphate-buffered 4% paraformaldehyde-1% glutaraldehyde, postfixed in 2% osmium tetroxide, dehydrated in graded ethanol, and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined by a Hitachi 7S electron microscope.

Immunoblot studies

Cells were washed in cold PBS and suspended in a high salt, detergent solution (0.6M KCl and 1% Triton X100 in PBS) on ice. After incubation for 30 min, the suspension was centrifuged for 30 min at 10,000g. The pellet, crude cytoskeletal sample, was used for sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE and Western blotting were done by standard procedures (Laemmli, 1970 and Towbin et al, 1979). The nitrocellulose blots were stained by anti-GFAP antibodies, using indirect immunoperoxidase method.

Transplantation

$1x10^6$ cells were inoculated into 13 adult Syrian golden hamsters (Japan SLC, Shizuoka, Japan) subcutaneously or intracranially. Tumors produced were fixed in 70% ethanol, dehydrated and embedded in paraffin. Sections were stained with hematoxylin and eosin or subjected to immunohistochemical studies. In some cases, tumors were aseptically excised into fragments and seeded on plastic dishes for culture.

Results

A hamster medulloblastoma induced by the Tokyo-1 strain of JC virus and its stable line in early passages were immunohistochemically negative for GFAP (Nagashima et al,

1984).

However, two years after the initiation of cell cultivation, when the present study started, approximately one third of the cell population was found to be stained by monoclonal antibody to GFAP. Accordingly, cell cloning was attempted and six independent clones positive for GFAP were obtained. One clone, referred to as I-23, was further characterized and described below.

Morphology

I-23 cells were of triangular or multiangular shape and rich in cytoplasm (Fig.1). There was no difference in morphology between I-23 cells and the parent cells in early passage. Electron microscopic examination revealed abundant fine fibrils in the cytoplasm with an average diameter of 8nm (Fig.2).

Immunocytochemical studies

All of the I-23 cells were stained by either monoclonal or polyclonal antibody to GFAP (Fig.3). Reticular staining was conspicuous throughout the cytoplasm, with a pronounced perinuclear deposition of reaction product. All the cells were also positive for vimentin with a similar staining pattern. I-23 cells were, however, negative for each of the neurofilament triplet proteins, NSE, S-100 protein, GS and also negative without primary antibodies as control study. I-23 cells showed a nuclear staining typical for papovavirus

T antigen, when overlaid with hamster serum containing antibody to SV40 T antigen (Fig.4).

Immunoblot studies

Crude cytoskeletal preparation from I-23 cells was examined for the presence of GFAP. Immunoblotting study using antibody to GFAP clearly demonstrated a band with an Mr of 47K daltons (Fig.5), which corresponds to that of hamster brain GFAP.

Transplantation studies

Tumors were produced in all hamsters 3 to 9 weeks after the subcutaneous inoculation of I-23 cells. Intracranial inoculation of the cells also produced circumscribed, solid tumors in the brain. Histological examination revealed that the overall appearance of the tumors resembled that of human fibrillary astrocytoma (Fig.6). By immunohistochemistry, the tumor cells were positive for GFAP (Fig.7) and vimentin. Tumor cells were found to grow very rapidly in culture and it was easy to establish stable cell lines. Cultured cells from these cell lines were similar to I-23 cells in morphology and were positive for GFAP. They showed nuclear staining for papovavirus T antigen. These cells produced again tumors in hamsters with similar histology to that produced by I-23 cells.

Discussion

A GFAP positive clone(I-23) was established from a cell line

derived from a GFAP negative hamster medulloblastoma induced by JC virus. It is clear that I-23 cells produce GFAP, because they can be stained by both monoclonal and polyclonal antibodies to GFAP and they show, in an immunoblot assay, a protein band with an Mr of 47Kdaltons, which corresponds to that of hamster brain GFAP. It is supported that GFAP produced in these cells is associated with cellular cytoskeleton by the reticular pattern of immunostaining and the presence of the 47K-dalton band in the crude cytoskeletal preparation of the cells. It is likely that GFAP is colocalized with vimentin in the intermediate filaments, which are abundant in their cytoplasm(Sharp et al, 1982, Schiffer et al, 1986, Roessmann et al, 1983, Yung et al, 1985).

A portion of the cultured cells from human GFAP positive astrocytoma generally, expresses GFAP in the early passages. Such cells, however, tend to disappear because of either dilution through passage or loss of phenotypic expression in culture(Vidard et al, 1978 and Osborn et al, 1981). It is, therefore, usually difficult to obtain GFAP positive cell lines from human astrocytoma(Bigner et al, 1981). I-23 cells are peculiar in that they have apparently acquired GFAP expression during a long term culture of GFAP negative medulloblastoma cells. Since I-23 cells express T antigen for papovavirus as determined here and harbor complementary DNA to JC viral DNA(Matsuda, personal communication), it is possible that GFAP positive cells were not contaminants

during passage, but derived directly from GFAP negative medulloblastoma cells which were transformed by the virus. Although a clear explanation of the occurrence of GFAP in these cells is difficult to draw, it is of interest to note that human medulloblastoma has been suggested to possess a potential to undergo either neuronal, ependymal or glial differentiation (Rorke 1983). It may be possible that some of the original hamster medulloblastoma cells transformed by the Tokyo-1 strain of JC virus had a potential of astrocytic differentiation. This view may be supported also by the recent studies that the Tokyo-1 strain of JC virus infects and transforms the bipotential cells in the cerebellar external layer, which may become medulloblastoma after migrating to the internal layer (Matsuda et al, 1987).

I-23 cells possessing GFAP expression were found to be highly transplantable. Histology of tumors produced in hamster by these cells were strikingly different from the original medulloblastoma, resembling that of human fibrillary astrocytoma (Eng et al, 1978, Osborn et al, 1983, Rungger et al, 1983). The tumor cells retained immunoreactivity to GFAP. I-23 cells, thus, appear to possess stable astrocytic features with respect to GFAP expression and tumor histology. This cell line might provide a useful tool for not only the mechanism of GFAP production but also biological studies of astrocytoma.

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Legend to figures:

Fig. 1 I-23 cells. HE stain. x125 The cells are of multiangular in shape and rich in cytoplasm.

Fig. 2 I-23 cells. Electron micrograph. x20000 Fibrils with an average diameter of 8 nm are abundant in the cytoplasm.

Fig. 3 I-23 cells. Immunostaining with a monoclonal antibody to GFAP. x300 All the cells are positively stained, with a reticular pattern.

Fig. 4 I-23 cells. Immunostaining for SV40 T antigen. x270 The nuclei of all the cells are positively stained.

Fig. 5 Immunoblot using a monoclonal antibody to GFAP. A, Crude cytoskeletal preparation of I-23 cells; B, Hamster brain homogenate. Single band with an Mr. of 47K daltons is clearly seen in A, which corresponds to the major GFAP band observed in B.

Fig. 6 Histology of a subcutaneous tumor in hamster produced by the inoculation of I-23 cells. HE stain. x100

Fig. 7 Immunostaining for GFAP. Subcutaneous tumor as described in Fig. 6. x150 Most of the tumor cells are positively stained for GFAP.

Fig. 1

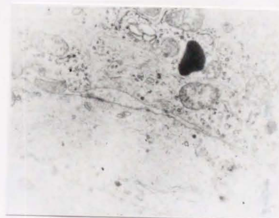


Fig. 2

Fig. 3

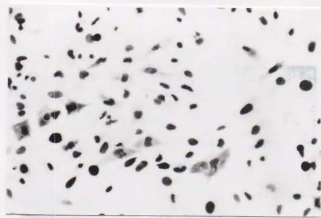


Fig. 4

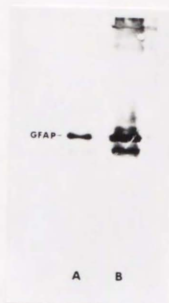


Fig. 5

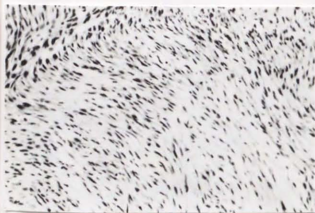


Fig. 6

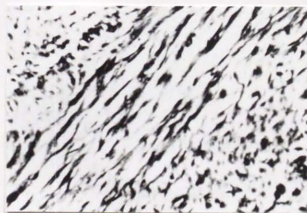
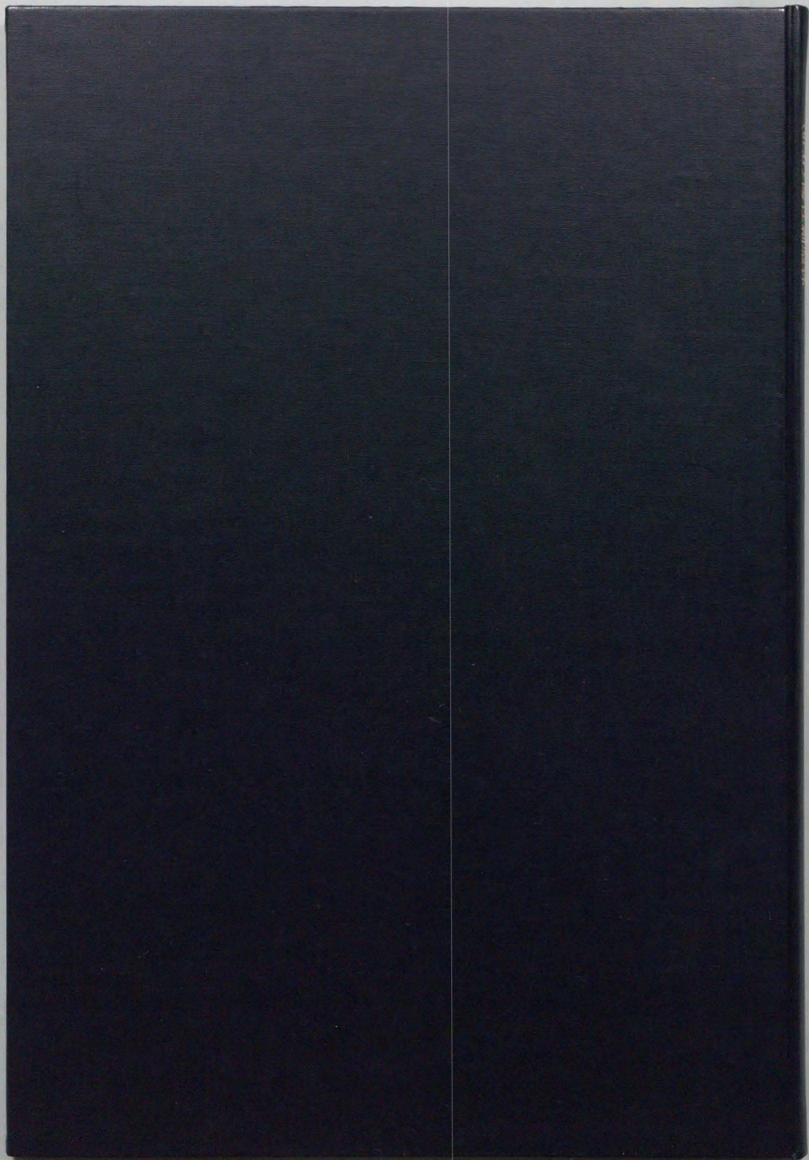


Fig. 7



Kodak Color Control Patches

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Blue Cyan Green Yellow Red Magenta White 3/Color Black

Kodak Gray Scale

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A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19