Original Article

Software-assisted morphometric and phenotype analyses of human peripheral blood monocyte-derived macrophages induced by a microenvironment model of human esophageal squamous cell carcinoma

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Human macrophages play important roles in tumor promotion and are called tumor-associated macrophages (TAMs). We previously demonstrated that human esophageal squamous cell carcinomas (ESCCs) contain TAMs and that these TAMs tend to have tumor-supporting features. Here we exposed human macrophages to conditioned media of TE-series human ESCC cell lines (TECMs) to generate an ESCC extracellular stimuli-influenced TAM model. CD14+ peripheral blood monocytes (PBMos) from healthy donors were treated with M-CSF and with additional IL-4 or TECM exposure. Morphological changes of the cells and the induction of CD163/CD204 proteins were detected in the TECM-exposed model TAMs by immunofluorescence. A software-assisted immunofluorescent cell image analysis showed increased CD163/CD204 positivity in the model TAMs and a weak to moderate positive correlation between the cytoplasmic area and the sum fluorescent intensity of CD204. Morphological changes of the cells were significantly reflected by several cytomorphometric parameters. PBMos were elongated with M-CSF treatment, then enlarged with TECM exposure. The cytoplasmic aspect ratio was decreased by M-CSF treatment and slightly increased by TECM exposure. The nuclear-cytoplasmic ratio decreased during the whole process of cell differentiation. This system is useful for quantitative assessments of TAM-like morphological changes of macrophages and the induction of CD163/CD204 in a model ESCC microenvironment.

Key words: esophageal cancer, image analysis, macrophages, tumor microenvironment

The tumor microenvironment consists of various components: neoplastic cells, microvessels, extracellular matrix, activated fibroblasts, and peripheral blood-derived immune cells including macrophages.1 A type of macrophage that has been shown to play an important role in tumor promotion is called the tumor-associated macrophage (TAM).2 Human macrophages are proposed to differentiate into pro-inflammatory (M1, classical) and/or anti-inflammatory (M2, alternative) phenotypes, and it has been shown that TAMs tend to have M2-like features.2,3 Human ovarian cancer cell line-derived conditioned media were shown to activate macrophages alternatively (M2) with interleukin (IL)-10high/IL-12low cytokine characteristics.4 Monoclonal antibodies to CD163, a membrane protein that is a member of the scavenger-receptor cysteine-rich domain family, and monoclonal antibodies to CD204, a macrophage scavenger receptor, have been widely used as M2-activated macrophage markers.2,5,6

Using immunohistochemistry, we previously observed that human esophageal squamous cell carcinoma (ESCC) tissues contained CD204+ macrophages, and their numbers were correlated with clinicopathological factors and the prognosis of ESCC patients.7 In another study, we exposed human peripheral blood monocyte (PBMo)-derived macrophage colony-stimulating factor (M-CSF)-induced model macrophages to conditioned media of ESCC cell lines to...
generate model TAMs. These TAMs were confirmed to have M2-like features with an IL-10(high)/IL-12(low) mRNA expression profile, and we subjected them to a cDNA microarray analysis.\textsuperscript{8} The candidate genes related to cancer proliferation, invasion, migration and prognosis remain to be analyzed.\textsuperscript{9,10} We also observed that with conditioned media exposure, THP-1 human acute monocytic leukemia cell line-derived model macrophages adhered to the bottom of the culture dish, changed their size and shape, and developed protruding pseudopods.\textsuperscript{7} We then hypothesized that both functional and morphological changes of macrophages occur during their differentiation into TAMs.

Based on these prior findings, we conducted the present study to investigate whether macrophages acquire both functional and morphological characteristics of TAMs in a model ESCC microenvironment, and we performed a software-assisted cytometric analysis of the model ESCC microenvironment-exposed TAMs. We exposed human M-CSF-induced model macrophages to conditioned media and observed the chronological changes in the size, shape and surface marker proteins quantitatively by a semi-automatic image analysis.

MATERIALS AND METHODS

Cell cultures

Five ESCC cell lines (TE-8, TE-9, TE-10, TE-11 and TE-15) were obtained from the RIKEN BioResource Center (Tsukuba, Japan).\textsuperscript{10} The individuality of the TE series ESCC cell lines was confirmed by a short tandem repeat repeat analysis at RIKEN and at the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan).

We routinely maintained the cells in RPMI-1640 (Wako, Osaka, Japan) with heat-inactivated 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) and 1% antibiotic-antimycotic (Invitrogen, Carlsbad, CA, USA). We prepared the conditioned media of the TE-series ESCC cell lines (TECMs) by plating 5 × 10^6 cells in 10 mL of complete medium in 100-mm dishes for 24 h, thereafter changing the medium to complete Dulbecco’s modified Eagle medium (DMEM, Wako) supplemented with heat-inactivated 10% human AB serum (Lonza, Walkersville, MD, USA) instead of FBS. After 2–3 d, the supernatants were harvested, centrifuged and stored in aliquots at −80°C.\textsuperscript{11}

Macrophage cultures and treatment

The study was approved by the institutional review board. Peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteer donors who provided written informed consent. CD14\textsuperscript{+} PBMos were purified from these PBMCs by positive selection using the auto MACS Pro Separator (Miltenyi Biotec, Bergisch Gladbach, Germany). PBMos were cultured with M-CSF (25 ng/mL; R&D Systems, Minneapolis, MN, USA) for 6 d to induce macrophage differentiation and additional recombinant human interleukin-4 (rhIL-4; 20 ng/ mL; R&D Systems) overnight to induce M2-polarization as described with some modification.\textsuperscript{12} We also added 50% TECMs for 2 d to the cultures of M-CSF-induced model macrophages to induce model TAMs as described.\textsuperscript{6} The viability of these M-CSF-treated and TECMs-exposed cells in the observation fields were confirmed to be more than 95% by Live/Dead Cell Staining Kit II (PromoKine, Heidelberg, Germany).

Transmitted light microscopy

Samples were observed with transmitted light by using a FLoid Cell Imaging Station (Life Technologies, Carlsbad, CA, USA; total magnification 460×). We captured cell images from the samples and then manually measured the maximum cell lengths of 10 representative cells including pseudopods from each image by using the ruler tool of GIMP 2.8.10 software (The GIMP team, http://www.gimp.org).

Double immunofluorescence

For double immunofluorescence, cells were cultured with Lab-Tek II 4-well chamber slides (Thermo Scientific, Waltham, MA, USA) and fixed with methanol (Wako, purity > 99.8%) at 4°C for 15 min. We used mouse monoclonal antibodies to CD68 (Kp-1, 1:100; DakoCytomation, Glostrup, Denmark), CD163 (10D6, 1:100; Leica Biosystems, New- castle upon Tyne, UK) or CD204 (SRA-E5, 1:25; Trans Genic, Kobe, Japan) mixed with rabbit monoclonal antibody against β-actin (13E5, 1:200, Cell Signaling Technology, Danvers, MA, USA) for the primary antibodies and incubated the cells at 4°C overnight. We used Alexa Fluor 488-conjugated anti-rabbit IgG and Cy3-conjugated anti-mouse IgG (1:200, Jackson ImmunoResearch, West Grove, PA, USA) for the secondary antibodies and incubated the cells at room temperature for 1 h. The nuclei were stained with DAPI (Wako). The samples were washed three times after each step and aqueously mounted with Dako Fluorescent Mounting Medium (Dako).

Image acquisition

We observed the samples with a BX50 fluorescence microscope (Olympus, Tokyo, Japan; total magnification 200×). A
DP73 digital camera with cellSens Standard 1.6 software (Olympus) was used for image capturing. Twenty immunofluorescent images (each was 1600 × 1200 pixels) per sample were manually obtained with no overlap among fields at 2, 4, and 6 d of M-CSF treatment and after additional TECM exposure (8 d). Black balance was realized automatically in a field without cells. To obtain a sufficiently dark background, the exposure time of each channel was set to 5 ms (blue), 150 ms to 1 s (green, adjusted to cytoplasmic β-actin fluorescent intensity and specimen moisture) and one-fifth of the green channel (red), respectively. The camera settings were saved and reused for all slides of the study.

Image processing and analysis

Immunofluorescent images were converted to 8-bit single channel images by ImageJ 1.47 software (National Institutes of Health, Bethesda, MD, USA). The 8-bit images were analyzed semi-automatically by TissueQuest 4.0 software (TissueGnotics, Vienna, Austria).13-15 The field of view (FOV) size was set at 0.898789346 × 0.66117837 mm for each image. We chose the Various Shapes 2.0 (TissueGnotics) segmentation method (an algorithm for the recognition of individual cells) and analyzed only cells entirely inside the FOV. We set markers for β-actin (cytoplasm), M2 marker proteins (CD163 or CD204) and DAPI (nuclei, as a master marker), and then configured the marker-dependent parameters. A marker in this context means a color-labeled cellular component used to identify individual cells. More precise experiment marker settings including the segmentation method parameters are shown in the supplemental Table S1. Using a raw data export module, we extracted the results from the analysis project to Excel 2013 software.

There were several parameters with specific meanings in the results. The mean intensity was defined as the average gray level expressed by the pixels of each object, and it indicates the intracellular concentration of a certain molecule in this context. The sum intensity was the sum of all gray-level intensities expressed by the pixels of each object; it indicates the intracellular total accumulation amount of a molecule. The object length was defined as the maximum length taking into account all possible measurement positions. The object width was defined as the minimum width. The object area was the area of the recognized segmented object (i.e., the nucleus or cytoplasm of each cell).

Statistical analysis

The statistical significance of differences was evaluated by paired t-test. P-values < 0.05 were considered significant. The strength of correlations is indicated as scarce (no statistical significance of differences), very weak (< 0.2), weak (0.2–0.4) or moderate (0.4–0.7). We also calculated Pearson correlation coefficients for scatter diagrams. Statistical analyses were carried out using SPSS Statistics ver. 22 (IBM, Chicago, IL, USA).

RESULTS

Both the IL-4 and TECM exposure promoted morphological changes of PBMo-derived macrophages

To generate a simple model of TAMs in ESCC tissue, we performed an IL-4 or TECM exposure assay. We cultured PBMos for 6 d with rhM-CSF (25 ng/mL) to induce model macrophages. The cells were then exposed to rhIL-4 (20 ng/mL) overnight to induce model M2 macrophages. Model macrophages were also exposed to TECM for an additional 2 d to induce model TAMs (Fig. 1a). The cells were almost round and floating at first; then they attached to the bottom of the culture dish, and pseudopods protruded after 6-d treatment with rhM-CSF and enlarged after the exposure to IL-4 or TECM (Fig. 1b). Figure 1c demonstrates the maximum cell lengths of 10 representative cells, which were manually measured from these images. The cells were significantly elongated with M-CSF treatment for 2–6 d (P < 0.001). Moreover, both the additional IL-4 and TECM-exposed cells were significantly elongated with IL-4, TE-8 CM, TE-11 CM and TE-15 CM exposure (P < 0.001), and with TE-9 CM and TE-10 CM exposure (P < 0.01).

TECM-exposed macrophages showed increased mean fluorescent intensities of M2 marker proteins (CD163/CD204) in the cell image analysis

Based on our observation of the morphological changes of macrophages, we hypothesized that soluble factor(s) from ESCC cells induce model macrophages to become TAM-like cells. We subsequently performed an immunofluorescence evaluation of M-CSF-treated TECM-exposed cells. Model macrophages were exposed to TECM for an additional 2 d. We conducted the double immunofluorescence of β-actin and macrophage markers, and microscopic images were acquired (Fig. 2a). CD68 and M2 marker proteins were expressed in the TAM-like morphologically changed cells with TECM exposure (Fig. 2b). The software-assisted cell image analysis demonstrated that the average CD163/CD204 mean intensity for each cell (Fig. 2c) showed a significant increase with TECM exposure (P = 0.001–0.015 for CD163, P = 0.021–0.035 for CD204). Interestingly, the average CD204 mean intensity also showed a significant increase over 2 d to 6 d (P = 0.037).
The cell image analysis revealed that TECM-exposed TAM-like cells had an increased fraction of M2 marker-positive cells

To quantitatively assess the effect of TECM exposure on macrophages, we conducted further cell image analyses. As described above, PBMos were cultured with rhM-CSF and induced model macrophages were exposed to TECM; immunofluorescent images were then acquired. The M2 marker proteins CD163 and CD204 were expressed in TECM-exposed TAM-like cells. Histograms in Figure 3 show the percentages of M2 marker-positive cells. The percentages of the CD163+ cells, which were <5% in the 6-d macrophages, were increased to 18%–72% with TECM exposure. The percentages of the CD204+ cells, which were 7%–13% in the 6-d macrophages, were increased to 23%–69% with TECM exposure.

There were positive correlations between the macrophage cytoplasmic area and the sum fluorescent intensity of M2 markers, especially that of CD204

To determine the correlations between the morphological changes and the intensity of M2 marker expression, we calculated the Pearson correlation coefficients. As shown in Figure 4, the scatter diagrams of the cytoplasmic area of the TAM-like cells and the CD163 sum intensity showed scarce (no significant differences, black), very weak (<0.2, green) or weak (0.2–0.4, orange) positive correlations between the cytoplasmic area and the sum intensity, with \( r = 0.002–0.227 \), whereas the CD204 sum intensity showed a weak (0.2–0.4, orange) to moderate (0.4–0.7, red) positive correlation with \( r = 0.241–0.565 \).

The cytomorphometric parameters significantly reflected the morphological changes of the model macrophages and TAMs

We also observed that the cytoplasmic and nuclear areas, the maximum lengths and the minimum widths of each cytoplasm or nucleus, the cytoplasmic aspect ratio (CAR) and the nuclear:cytoplasmic (N:C) ratio significantly reflected the morphological changes of the model macrophages and TAMs. We plotted the means of these parameters’ values in Figure 5 to clarify how these morphological parameters shift as PBMo-derived cells differentiate with M-CSF treatment and additional TECM exposure. We used the data of two
sample groups per donor \((n = 6)\). The mean cytoplasmic length (Fig. 5a) was increased compared to the mean cytoplasmic width (Fig. 5b) from 2 d to 6 d, indicating cytoplasmic elongation. After the exposure to TECM, the mean cytoplasmic area increased significantly (Fig. 5c, \(P < 0.001\)) along with the significant increases in cytoplasmic length and width.

The means of nuclear parameters (Fig. 5d–f) showed no significant changes at 2–6 d \((P = 0.219–0.960)\), but they increased significantly after the exposure to TECM, which indicates nuclear enlargement \((P < 0.001)\). The mean CAR (Fig. 5g) significantly decreased from 2 to 6 d \((P < 0.001)\) and slightly but significantly increased with TECM exposure \((P < 0.05)\). The mean N:C ratio decreased significantly during the whole process of macrophage differentiation (Fig. 5h,i, \(P < 0.01\)).

Our proposed model of morphological changes of the cells is illustrated in Figure 6. The mean CAR was approx. 1.0 in the PBMs. After M-CSF treatment, the cytoplasm was elongated and the mean CAR dropped to approx. 0.5 in the model macrophages. With additional TECM exposure, both the cytoplasm and nuclei were enlarged and the mean CAR recovered to approx. 0.6 in the model TAMs.

**DISCUSSION**

Over the past few decades, a considerable number of studies have been conducted to visualize the interactions between cancer cells and macrophages.\(^\text{16,17}\) There has also been growing interest in the morphological changes of...
Figure 3  Immunofluorescent images and the M2 marker intensity histograms of three samples. M2 marker proteins (red) were expressed in tumor-associated macrophage (TAM)-like cells. β-actin (green) refers to cytoplasm. Scale bar, 50 μm. Histograms show the percentages of M2 marker-positive cells. The percentages of M2 marker-positive cells were increased with TE-series ESCC cell line-conditioned medium (TECM) exposure. The x- and y-axes show the mean intensity of the M2 markers and the number of cells for each sample, respectively.

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Figure 4  Scatter diagrams of cytoplasmic area and the M2 marker sum intensity of three samples. The x- and y-axes show the cytoplasmic area (μm²) and the M2 marker sum intensity for each sample, respectively. Pearson correlation coefficients and the statistical significance of differences were calculated for each sample. There were weak to moderate positive correlations between the macrophage cytoplasmic area and the sum intensity of the M2 markers, especially that of CD204. The strength of the correlations is indicated as follows: moderate (0.4–0.7, red), weak (0.2–0.4, orange), very weak (< 0.2, green) or scarce (no significant differences, black). *P < 0.05, **P < 0.01.
Figure 5 Means of cytoplasmic and nuclear morphometric parameters of peripheral blood monocyte (PBMo)-derived M-CSF-treated cells and additional TE-series ESCC cell line-conditioned medium (TECM)-exposed cells. The morphological changes of the PBMo-derived cells reflected the cytoplasmic aspect ratio and N:C ratio. (a–c) Cell images were obtained 2, 4, and 6 d and after additional TECM exposure (8 d). The cytoplasmic length, width and area of each cell were calculated automatically by TissueQuest software. The mean cytoplasmic length (a), width (b) and area (c) of each sample are shown. (d–f) The nuclear length, width and area of each cell were calculated in a similar way. The mean nuclear length (d), width (e) and area (f) of each sample are shown. (g) The cytoplasmic aspect ratio (CAR) of each cell was automatically calculated by dividing the cytoplasmic width by the cytoplasmic length. The mean CAR of each sample is shown. (h) The N:C ratio (length) of each cell was automatically calculated by dividing the nuclear length by the cytoplasmic length. The mean N:C ratio of each sample is shown. (i) The N:C ratio (area) of each cell was calculated in a similar way (nuclear area divided by cytoplasmic area). n = 6 (two sample groups per donor). *P < 0.05, **P < 0.01, ***P < 0.001. Sample 1-1, Sample 1-2; Sample 2-1, Sample 2-2; Sample 3-1, Sample 3-2.
macrophages during their differentiation process. In the 1970s, Zuckerman et al. carried out long-term human PBMo cultures and observed the morphological changes of PBMos into macrophages with successive measurements.\textsuperscript{18} McWhorter et al. showed that forced macrophage elongation itself also modulates the macrophage phenotype in mouse bone marrow-derived macrophages.\textsuperscript{19}

In terms of cancer research, several studies using image analyses in a human breast cancer cell line-injected mouse \textit{in vivo} xenograft model have been reported.\textsuperscript{20,21} However, these researchers focused mainly on the macrophage population density or protein expression. To our knowledge, there are no prior reports of a software-assisted analysis of the morphological changes and protein expression of an individual human TAM model.

We previously performed several studies using ESCC TAM models in which PBMos or THP-1 human acute monocytic leukemia cell line-derived macrophages were exposed to TECMs, and the results confirmed the induction of the mRNA expression of CD163/CD204 (for PBMo-derived cells) and CD204 (for THP-1 derived cells).\textsuperscript{7–9} We subsequently adopted this TECM exposure assay to a cell image analysis because using conditioned media is one of the most common and accessible methods to simplify a humoral factor(s)-mediated tumor microenvironment model.\textsuperscript{4}

Since cancer cells and stromal cells directly contact each other \textit{in vivo}, we performed a direct co-culture assay of PBMos and TE cells, in which co-cultured model TAMs showed stellate-like morphological changes and CD68+/CD163+/CD204+ by immunofluorescence (Fig. S1). We also confirmed the existence of CD68+/CD163+/CD204+ elongated macrophages in the surgically resected ESCC tissue specimens (Fig. S2). From these findings, we considered that it was necessary to perform a more precise morphological study of the macrophage differentiation process into TAMs in an ESCC microenvironment.

In the present study, the macrophage cell image and the M2 marker intensity histogram showed an increased fraction of M2 marker-positive cells with TECM exposure. Scatter diagrams of the cytoplasmic area and M2 markers indicated that CD204 was induced in TAM-like expanded cells. Interestingly, CD204 was also expressed in pre-differentiated cells after 4–6 days of M-CSF treatment. These results suggest that CD204 was activated earlier during macrophage maturation and preserved its activity in TAMs longer than CD163.

The dramatic enlargement of the cytoplasm was related to structural changes of cytoskeleton molecules (including actin and microtubules). However, the potential inducer of macrophage morphological change itself remains still unclear. Recently, receptor tyrosine kinase family Mertk was reported as one of the key molecules of microtubule-organizing centers (MTOC) of mouse macrophages and its dysfunction was related to not only macrophage phagocytosis but also macrophage cell shape and migration.\textsuperscript{22} We are now aiming at the analysis of the MTOC modulating factors including MERTK. Our system would be applicable to the objective interpretation of these molecules in the future. Interestingly, the nuclei had also enlarged, which may indicate nuclear activation and structural change;\textsuperscript{23} however, the underlying molecular mechanism in macrophages has not been well studied yet.

The greatest advantage of applying the software-assisted immunofluorescent cell image analyses to an ESCC microenvironment model is that we can analyze very large populations of cells (thousands of cells in a sample), including cells on their way to differentiating into macrophages, providing data that are difficult to obtain from tissue specimens of human ESCC clinical cases. This method also enables us to approximate the concentrations and the accumulation amounts of the marker proteins in individual cells and cell populations.

There are several limitations in this study. The exact molecules in TECMs that affect the TAM-like differentiation of M-CSF-induced macrophages remain to be analyzed more precisely. The above-mentioned McWhorter et al. study also treated mouse bone marrow-derived macrophages with exogenous cytokines and demonstrated macrophage elongation with IL-4 + IL-13 or expansion with lipopolysaccharide + interferon-gamma.\textsuperscript{19} The human PBMos in our study showed similar morphological changes when treated with M-CSF followed by IL-4, which suggests that exogenous cytokine(s)-stimulated M2-polarized macrophages tend to elongate and to become spindle-like. Our model TAMs expanded similarly to M1-polarized macrophages in their model, which indicates that TECM contain more complicated factors. The entire M1/M2 marker expression profile remains to be clarified. Although our method should be applicable to...
various immunofluorescent cell images, we must identify the optimal conditions of staining, sample preservation and image acquisition to obtain reproducible data.

We found that PBMo-derived M-CSF-induced macrophages showed both M2 marker expression and morphological changes with TECM exposure, which could be clearly interpreted by the immunofluorescent cell image analysis. Our findings also revealed the heterogeneity of the PBMo-derived cell population. This system may be useful for the quantitative assessment and objective interpretation of TAM-like morphological changes of macrophages in model ESCC microenvironments, and it could also be a useful experimental model for analyzing the mechanisms by which macrophages differentiate into TAMs in human ESCC tissues.

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DISCLOSURE STATEMENT

None declared.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site.
Table S1 Option parameters for TissueQuest® experiment marker settings.

Figure S1 Co-culture assay of PBMos with ESCC cells promotes M2 differentiation.

Figure S2 CD68+/CD163+/CD204+ elongated cells are contained in the surgically resected ESCC tissues.