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Concise report

Identification of relapse predictors in IgG4-related disease using multivariate analysis of clinical data at the first visit and initial treatment

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Abstract

Objectives. Inducting clinical remission by glucocorticoid treatment is relatively easy in IgG4-related disease (IgG4-RD), but relapse also occurs easily with tapering of the steroid dose. The present study tried to analyse the cases to extract predictors of relapse present at the diagnosis of IgG4-RD.

Methods. Subjects comprised 79 patients with IgG4-related dacryoadenitis and sialadenitis, known as Mikulicz's disease, who were diagnosed between April 1997 and October 2013 and followed-up for >2 years from the initial induction treatment. They were applied to Cox proportional hazard modelling, based on the outcome of interval to relapse. We performed multivariate analysis for the clinical factors of these cases and identified predictors of relapse.

Results. Identified factors were male sex and younger onset in cases without organ involvement at diagnosis and low levels of serum IgG4 in cases with organ dysfunction at diagnosis. Complication with autoimmune pancreatitis and low steroid dose at initial treatment also tended to be associated with recurrence.

Conclusion. Follow-up is important in cases with recognized risk factors for relapse, including male sex and younger onset in cases without organ damage.

Key words: autoimmune pancreatitis, IgG4-related disease, Mikulicz's disease, multivariate analysis, relapse.

Introduction

IgG4-related disease (IgG4-RD) can cause irreversible damage to various organs through type 2 T helper (Th2) inflammation and progressive fibrosis [1, 2]. Induction of clinical remission is easily achieved using glucocorticoid treatment [3], but relapse also readily occurs when the steroid dose is tapered [4]. The annual rate of recurrence in 2012 was 19.0% in our facility, and half of relapsed cases reportedly present with new organ lesions [5]. On the other hand, many cases can continue in clinical remission with low-dose glucocorticoid, and steroid can even be discontinued in some cases. Because no markers can reflect disease activity and predict relapse in IgG4-RD, rheumatologists often encounter difficulties in clinical practice. Thus the present study tried to analyse cases followed for >2 years after initiating therapy to extract predictors of relapse present at the diagnosis of IgG4-RD.

Methods

Subjects comprised 79 patients with IgG4-related dacryoadenitis and sialadenitis, known as Mikulicz's

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CLINICAL SCIENCE

disease, who were diagnosed between April 1997 and October 2013 and followed up for >2 years from the initial induction treatment. Cases were diagnosed with bilateral and continuous enlargement of the lacrimal and salivary glands, elevated levels of serum IgG4 and abundant infiltration of IgG4-bearing plasmacytes into involved organs. We analysed the following clinical factors: sex; age at onset; disease duration; eosinophil count; serum levels of IgG, IgG4 and IgE at diagnosis; presence of hypocomplementaemia and ANA and levels of RF at diagnosis; presence of organ involvement other than the lacrimal and salivary glands; numbers of organ lesions other than those of the lacrimal and salivary glands; complication with autoimmune pancreatitis, IgG4-related kidney disease or retroperitoneal fibrosis and the initial dose of steroid. Disease duration was defined as the interval between the appearance of subjective symptoms and the start of treatment. Autoimmune pancreatitis, IgG4-related kidney disease and retroperitoneal fibrosis were diagnosed based on imaging findings. As our treatment protocol, starting prednisolone at a dose of 0.6 mg/kg/day was appropriate with only lacrimal and salivary gland involvement, increasing to 1.0 mg/kg/day with multiple organ lesions. The initial dose of prednisolone was continued for 2–4 weeks, tapering the dose by 10% every 2 weeks. If the patient was >80 years of age or had existing complications, the amount of prednisolone was decreased up to 30% of the predetermined amount. Relapse was defined as re-enlargement of the lacrimal and/or salivary glands or appearance of other organ involvement.

First, all 79 cases were applied to Cox proportional hazard modelling, based on the outcome of interval to relapse. We performed uni- and multivariate analysis for each clinical factor and identified predictors of relapse. On multivariate analysis, we used the backward elimination method (Wald method, excluding factors presenting with $P > 0.1$) and extracted the factors offering high predictive power. The existence of organ involvement was considered to represent a strong risk factor for relapse. We stratified patients into groups with and without organ lesions at diagnosis and performed uni- and multivariate analysis for each group. In multivariate analysis, we applied those variables that showed $P < 0.2$ in univariate analysis and used the backward elimination method (Wald method, excluding factors presenting with $P > 0.1$). P-values < 0.05 were considered statistically significant. All statistical analyses were performed using SPSS Statistics version 20.0.0 software (IBM, Armonk, NY, USA).

Written consent to use the information from these cases was obtained from all patients in accordance with the Declaration of Helsinki. This study proceeded under the approval of the Sapporo Medical University Hospital Institutional Review Board (SMU 22-57, 24-155).

Results

Table 1 shows the profiles of the patients. For the 79 cases, age at onset, presence of organ involvement and complication of autoimmune pancreatitis at diagnosis in univariate analysis and age at onset, levels of serum IgG and RF and presence of organ involvement at diagnosis in multivariate analysis were extracted.

The results from analyses by group with and without organ involvement, which was considered a strong predictor of relapse, showed that sex, age at onset and disease duration were significant on univariate analysis and sex and age at onset were extracted from multivariate analysis for the group without organ lesions at diagnosis. On the other hand, levels of serum IgG and IgG4 at diagnosis were significant on univariate analysis and the level of serum IgG at diagnosis was extracted by multivariate analysis for the group with organ dysfunction (Table 2).

TABLE 1 Characteristics of the patients

	Organ involvement at the first visit			
	Presence		Absence	
	No relapse during follow-up $n = 28$	Relapse during follow-up $n = 5$	No relapse during follow-up $n = 24$	Relapse during follow-up $n = 22$
Male:female, mean (S.D.)	8:20 (1:2.5)	3:2 (1:0.7)	16:8 (1:0.5)	10:12 (1:1.2)
Age at onset, mean (S.D.), years	62.6 (10.8)	45.8 (18.0)	60.3 (8.5)	55.2 (12.7)
Period of illness, mean (S.D.), years	1.39 (1.69)	4.00 (3.39)	1.29 (1.81)	2.45 (2.63)
Eosinophils, mean (S.D.), per ml	220.7 (252.8)	106.0 (60.7)	234.4 (195.5)	314.3 (287.8)
Serum IgG, mean (S.D.), mg/dl	2035.0 (1027.0)	1479.6 (160.2)	3326.3 (2338.5)	2264.5 (880.4)
Serum IgG4, mean (S.D.), mg/dl	556.5 (393.2)	221.8 (181.1)	1192.1 (1033.4)	716.7 (466.8)
Serum IgE, mean (S.D.), IU/ml	314.1 (439.4)	412.4 (395.5)	470.8 (391.9)	272.1 (241.3)
Hypocomplementaemia, %	14.3	20.0	29.2	18.2
ANA positive, %	17.9	40.0	25.0	18.2
RF positive, %	14.3	60.0	16.7	18.2

Table 2 Uni- and multivariate analysis for risk factors associated with relapse in IgG4-related disease

	Univariate analysis			Multivariate analysis		
	P-value	Hazard ratio	95% CI	*P*-value	Hazard ratio	95% CI
Totals ($n = 79$)						
Male sex	0.120	1.869	0.850, 4.109			
Age at onset	**0.006**	**0.961**	0.935, 0.989	**0.004**	**0.955**	0.925, 0.985
Disease duration	0.062	1.119	0.994, 1.261			
Eosinophil count	0.641	1.000	0.998, 1.001			
Serum level of IgG before Tx	0.052	1.000	0.999, 1.000	**0.003**	**0.999**	0.999, 1.000
Serum level of IgG4 before Tx	0.111	0.999	0.999, 1.000			
Serum level of IgE before Tx	0.180	0.999	0.998, 1.000	0.051	0.999	0.997, 1.000
Hypocomplementaemia	0.312	0.602	0.225, 1.612			
ANA	0.707	0.839	0.337, 2.093			
RF	0.575	1.281	0.540, 3.042			
RF titre	0.743	0.998	0.989, 1.008	**0.010**	**1.018**	1.004, 1.033
Other organ involvement	**0.010**	**3.590**	1.355, 9.512	**<0.001**	**11.077**	3.028, 40.523
Number of other organ involvements	0.122	1.340	0.925, 1.940			
Autoimmune pancreatitis	**0.013**	**3.184**	1.280, 7.924			
IgG4-related kidney disease	0.953	0.968	0.333, 2.813			
Retroperitoneal fibrosis	0.447	1.379	0.603, 3.151			
Initial dose of glucocorticoid	0.595	1.009	0.977, 1.041	0.050	0.953	0.908, 1.000
Without organ involvement ($n = 33$)						
Male sex	0.078	7.842	0.796, 77.228	**0.015**	**342.461**	3.069, 38217.013
Age at onset	**0.022**	**0.938**	0.887, 0.991	**0.004**	**0.856**	0.769, 0.952
Disease duration	**0.008**	**1.554**	1.122, 2.152			
Eosinophil count	0.215	0.994	0.984, 1.004			
Serum level of IgG before Tx	0.115	0.996	0.992, 1.001			
Serum level of IgG4 before Tx	0.116	0.995	0.989, 1.001			
Serum level of IgE before Tx	0.996	1.000	0.998, 1.002			
Hypocomplementaemia	0.958	0.941	0.102, 8.715			
ANA	0.231	3.015	0.496, 18.334			
RF	0.108	4.397	0.724, 26.721			
RF titre	0.881	0.998	0.967, 1.029			
Initial dose of glucocorticoid	0.320	1.094	0.916, 1.306			
With organ involvement ($n = 46$)						
Male sex	0.928	1.041	0.441, 2.453			
Age at onset	0.152	0.974	0.939, 1.010			
Disease duration	0.882	1.011	0.875, 1.168			
Eosinophil count	0.978	1.000	0.998, 1.002			
Serum level of IgG before Tx	**0.012**	**0.999**	0.999, 1.000	**0.015**	**0.999**	(0.999, 1.000)
Serum level of IgG4 before Tx	**0.023**	**0.999**	0.999, 1.000			
Serum level of IgE before Tx	0.142	0.999	0.997, 1.000			
Hypocomplementaemia	0.204	0.491	0.164, 1.472			
ANA	0.148	0.438	0.144, 1.338			
RF	0.933	0.954	0.319, 2.851			
RF titre	0.800	0.999	0.990, 1.007			
Number of other organ involvements	0.375	0.732	0.367, 1.459			
Autoimmune pancreatitis	0.089	2.417	0.875, 6.673	0.054	2.828	(0.981, 8.157)
IgG4-related kidney disease	0.337	0.587	0.198, 1.741			
Retroperitoneal fibrosis	0.553	0.767	0.320, 1.842			
Initial dose of glucocorticoid	0.127	0.974	0.941, 1.008	0.055	0.960	0.921, 1.001

Tx: treatment. Significant values are indicated in bold. The upper section of the table shows total, the middle section shows without organ involvement other than lacrimal and salivary gland lesions and the lower section shows with organ involvement other than lacrimal and salivary gland lesions.

Male sex and younger onset in cases without organ involvement and a low level of serum IgG at diagnosis in cases with organ dysfunction were identified as predictors of relapse.

Discussion

The results of this analysis showed that extracted predictors of relapse differed between cases with

and without organ dysfunction at diagnosis in IgG4-related dacryoadenitis and sialadenitis. This might provide an opportunity to reconsider initial treatment in IgG4-RD.

First, we discuss cases without organ lesions other than of the lacrimal and salivary glands in IgG4-related dacryoadenitis and sialadenitis. Male sex and younger onset were predictors of relapse. IgG4-RD is a disorder based on Th2 inflammation [1]. With regard to the relationship between Th1/Th2 cytokine balance and sex hormones, oestrogen is known to promote the Th1 response [6], while progesterone promotes Th2 inflammation [7]. For this reason we have sometimes found that symptoms worsen when a female patient with IgG4-RD becomes pregnant. In addition, Th1 response is gradually suppressed in menopause due to the reduction in the production of oestrogen. In other words, Th2 immune response tends to be dominant in women after menopause. On the other hand, dihydrotestosterone, an active androgen, inhibits both Th1 and Th2 immune responses [8]. Th2 response is less likely to arise in males and younger patients due to the sex hormone environment. The occurrence of IgG4-RD in males and younger patients may thus suggest high disease activity. These results could also be confirmed using the SMART (Sapporo Medical University and related institutes database for investigation and best treatments of IgG4-RD) cohort database. We analysed 110 cases treated with maintenance therapy and overlapped them with the 79 subjects in the main analysis. The annual relapse rate in the male cases without organ involvement was 9.09% and in the female cases it was 3.85%. The amount of prednisolone at the maintenance treatment was 5.27 mg/day (S.D. 2.72) in males without organ involvement and 3.96 mg/day (S.D. 2.82) in females. Furthermore, there was no relapse in patients who were $\geqslant$70 years of age.

On the other hand, the low levels of serum IgG before treatment in cases with organ dysfunction are difficult to interpret. It was previously reported that expression levels of IL-6 mRNA at diagnosis of IgG4-RD were not significantly low [9]. The interpretation of this result is very difficult at present. In our analysis, complication with autoimmune pancreatitis and the use of low-dose glucocorticoid at initial induction therapy were not significant factors, but tended to be associated with relapse in cases with organ involvement. These factors might be identified as significant with increased numbers of cases for analysis.

The rate of complication with autoimmune pancreatitis was approximately 20% in these patients. Although this study could not suggest a precise interpretation, there was also a high rate of relapse in younger cases with autoimmune pancreatitis. This subject was not included in the cases with only autoimmune pancreatitis, and so was not statistically examined, but younger and male might be predictive risk factors in IgG4-RD.

There is currently no guideline on treatment in IgG4-RD as a whole. Japanese pancreatologists have developed a treatment guideline only for autoimmune pancreatitis. It recommends that the indication for steroid treatment is only symptomatic, starting at 0.6 mg/kg/day of prednisolone as initial dose [10]. This strategy can lead to clinical remission, but relapse often occurs. It is possible that the initial dose, which is required for the pathogenesis, is insufficient. Our analysis showed that the rate of recurrence was high in cases where we could not prescribe the predetermined amount and cases with multiple organ involvement.

We also have to follow up those cases with recognized risk factors for relapse, namely male sex and younger onset in the absence of organ damage. A sufficient dose of steroid at the initial induction treatment may inhibit recurrence in cases complicated with autoimmune pancreatitis.

Rheumatology key message

- Relapse predictors in IgG4-related disease without organ involvement at diagnosis were male sex and younger onset.

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Disclosure statement: The authors have declared no conflicts of interest.

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LETTER

Seasonal allergies and serial changes of serum levels of IgG4 in cases treated with maintenance therapy for IgG4-related disease

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Allergen, Glucocorticoid, IgE, IgG4, IgG4-related disease

History
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To the Editor,

Immunoglobulin G4-related disease (IgG4-RD) is a chromic and inflammatory disease that results in irreversible organ dysfunction in the long term due to helper T type 2 (Th2) inflammation and fibrosis [1]. Glucocorticoid treatment is useful for the induction of remission, but the disease tends to relapse upon tapering off of steroid treatment [2]. There is no biomarker for disease activity of IgG4-RD at this time. Tabata et al. reported that the serial changes of serum IgG4 levels could predict the recurrence of IgG4-RD [3], but in daily practice we have often seen relapse without elevation of serum IgG4 levels, as well as mild elevation of serum IgG4 levels without relapse. Meanwhile, an association of IgG4-RD with allergy has been suggested [4]. To address these issues, we analyzed the relationships between serum IgG4 levels and seasonal allergens in IgG4-RD. This retrospective study was performed in compliance with applicable ethical regulations, as certified by our hospital's Institutional Review Board.

The subjects were the 62 IgG4-RD cases who met the comprehensive diagnostic criteria for IgG4-RD [5]. The cases were sorted according to presentation in 2013 using the following classifications: no relapse; steroid dose changed by 2 mg/day or less; and no prescription of rituximab. We checked specific allergens before treatments, and measured the levels of serum IgG4 and IgE at each visit. We analyzed whether the peak levels of these two Igs coincided with allergen release (i.e., pollen scattering) periods. The specific allergens tested were as follows: seasonal allergens of *Betula verrucosa* (Bet v 1), *Cryptomeria japonica* (Cry j 1/Cry j 2), *Tinea translucens* (Tin t 1), *Dactylis glomerata* (Dac g 1), *Phleum pratense* (Phl p1/Phl p 12), *Anthoxanthum odoratum* (Ant o 1), *Ambrosia artemisiifolia* (Amb a 1), *Artemisia vulgaris* (Art v 1), *Leucanthemum vulgare* (Leu v 1), and perennial allergens of *Dermatophagoides pteronyssinus* (Der p 1/Der p 2), *Canis familiaris* (Can f 1), *Felis domesticus* (Fel d 1), *Candida albicans* (Can a 1), *Aspergillus fumigatus* (Asp f 1), and *Penicillium citrinum* (Pen c 3). The levels of serum IgG4 were measured by nephelometry; levels of IgE were measured by fluorescence-enzyme immunoassay; and levels of allergen-specific IgE were measured by radio-immunosorbent test. Serial changes of IgG4 and IgE were defined as fluctuations of over 20% from the mean concentration across all visits. Specific IgE concentrations of >0.70 UA/mL were defined as positive reactions for the allergens.

Seven (11.3%) of the 62 cases exhibited positive response for seasonal allergens only. These positive responses consisted of four cases harboring IgE for *B. verrucosa* (Bet v 1), and three harboring IgE for *D. glomerata* (Dac g 1). In Northern Japan (the location of this study), *B. verrucosa* pollen is released in May, while *D. glomerata* pollen is released between June and July. They showed clinical symptoms, including rhinorrhea and rhinostenosis in these seasons, but they were not prescribed with both anti-allergy medication and glucocorticoid. The levels of serum IgG4 in all of these cases were elevated in the respective seasons (Figure 1). Two of them showed IgE for several seasonal allergens, but it was considered that there was less effect on this analysis because the titers of IgE for Bet v 1 were significantly higher than those of the other allergens. On the other hand, twenty-five (40.3%) of the 62 cases exhibited serial changes of IgG4 levels. The fluctuation ranges were larger in proportion to the levels of serum IgG4 at the time of diagnosis. There were, of course, no relapsed cases. 25 cases with allergies to perennial allergens or without allergies did not show these serial changes. With regard to IgE, most cases (with or without allergies) exhibited less changes in IgE levels or discrepancy between the peak of IgE levels and the allergens-scattering period.

This study revealed that serum IgG4 levels also correlated with seasonal allergies in subset of cases with IgG4-RD.

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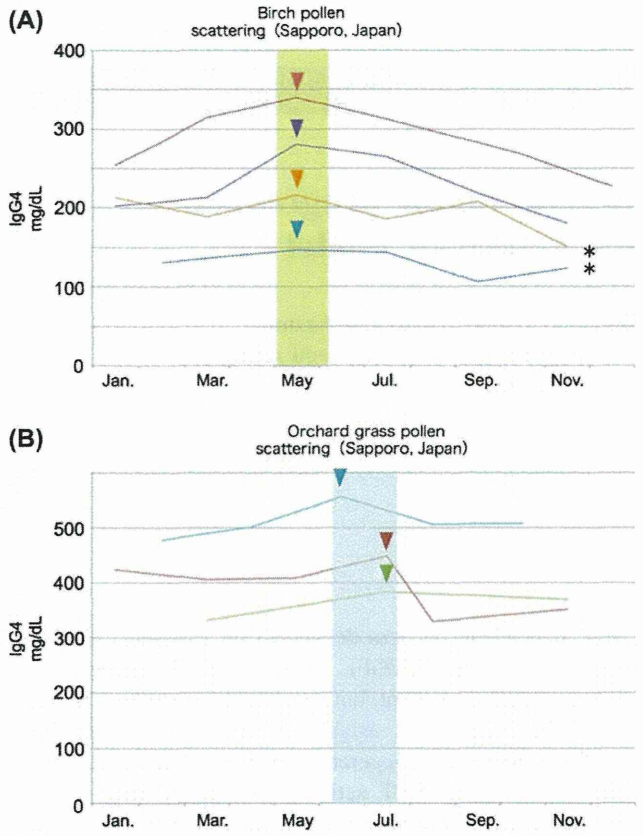

Figure 1. Seasonal changes of the levels of serum IgG4 in IgG4-related disease. (A) *B. verrucosa* pollen, (B) *D. glomerata* pollen. Serum IgG4 levels peaked during the pollen release periods of the seasonal allergens in the cases with the seasonal changes of serum IgG4 levels. The cases with asterisk had IgE for several seasonal allergens.

A quarter of the cases presented with allergies against both seasonal and perennial allergens; these cases therefore were excluded from this analysis. The final number of analyzable cases was small because of the exclusion of patients with low allergen titers, but we noted that the peak of serum IgG4 concentrations coincided with the scattering period of allergens in all cases that presented with serial changes in IgG4 levels and positive for IgEs against seasonal allergens. We hypothesize that seasonal allergies affected the production of IgG4 in these cases.

The physiology of IgG4 is still unknown, but this Ig has been proposed to be a blocking antibody for IgE [6]. We also measured serum IgE levels in the cases with seasonal allergies, but seasonal changes were not observed. The lack of seasonal changes likely reflects the vivo half-life time of IgE. The half-life of IgG4 is estimated as approximately 21 days, while that of IgE is only 2 days; thus levels of the latter are expected to decrease rapidly in the absence of exposure to allergens [7]. It is possible that peak IgE levels could not be accurately measured during the scattering periods of the allergens.

Terao et al. reported that serum IgG levels show seasonal changes in patients with autoimmune diseases [8]. It is unknown at this point whether the levels of serum IgG4 in healthy controls exhibit seasonal changes, but we found that serum IgG4 levels in some IgG4-RD cases with maintenance therapy or discontinuing steroid were transiently affected by the seasonal allergies. This phenomenon may complicate clinical evaluation, especially for cases where IgG4-RD disease activity is detected only by levels of serum IgG4. The clinical significance of the serial changes of serum IgG4 levels during treatment for IgG4-RD will require further investigation. Additional data will be needed to define useful biomarkers for IgG4-RD.

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Conflict of interest

None.

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ORIGINAL ARTICLE—ALIMENTARY TRACT

Contextual niche signals towards colorectal tumor progression by mesenchymal stem cell in the mouse xenograft model

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Abstract
Background The role of mesenchymal stem/stromal cells (MSCs) in tumorigenesis remains controversial. This study aimed to determine whether heterotypic interactions between MSCs and colon cancer cells can supply contextual signals towards tumor progression.
Methods Xenografts consisting of co-implanted human colorectal cancer cells with rat MSCs in immunodeficient mice were evaluated by tumor progression, angiogenic profiles, and MSC fate. Furthermore, we investigated how MSCs function as a cancer cell niche by co-culture experiments in vitro.
Results Tumor growth progressed in two ways, either independent of or dependent on MSCs. Such cell line-specific dependency could not be explained by host immune competency. COLO 320 xenograft angiogenesis was MSC-dependent, but less dependent on vascular endothelial growth factor (VEGF), whereas HT-29 angiogenesis was not MSC-dependent, but was VEGF-dependent. MSCs and COLO 320 cells established a functional positive feedback loop that triggered formation of a cancer cell niche, leading to AKT activation. Subsequently, MSCs differentiated into pericytes that enhanced angiogenesis as a perivascular niche. In contrast, the MSC niche conferred an anti-proliferative property to HT-29 cells, through mesenchymal–epithelial transition resulting in p38 activation.
Conclusions In conclusion, MSCs demonstrate pleiotropic capabilities as a cancer cell or perivascular niche to modulate colorectal cancer cell fate in a cell line-dependent manner in a xenogeneic context.

Keywords Mesenchymal stem cell · Niche · Pericyte · Cancer-associated fibroblast · Angiogenesis

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Abbreviations

MSCs	Mesenchymal stem/stromal cells
TME	Tumor microenvironment
CAFs	Cancer-associated fibroblastic cells
CCL5	C–C motif chemokine ligand 5
EMT	Epithelial–mesenchymal transition
IL-6	Interleukin-6
VEGF	Vascular endothelial growth factor
eGFP	Enhanced green fluorescence protein
αMEM	α-Modified Eagle's medium
FBS	Fetal bovine serum
qRT-PCR	Quantitative real-time reverse transcription PCR
MVD	Tumor microvessel density
Thy-1	Thymus cell antigen-1
NG2	Neural/glial antigen 2
αSMA	α-Smooth muscle actin
OE	Overexpression
CXCL12	Chemokine C-X-C motif ligand 12
KD	Knock down
MSC-CM	MSC-conditioned medium
CXCR4	C-X-C chemokine receptor type 4
MAPKs	Mitogen-activated protein kinases
FACS	Fluorescence-activated cell sorting
ANOVA	Analysis of variance
PECAM-1	Platelet endothelial cell adhesion molecule-1
Vegfr1 (Flt1)	Vascular endothelial growth factor receptor 1
PDGF-BB	Platelet-derived growth factor BB
Pdgfr-β	Platelet-derived growth factor receptor-β
MET	Mesenchymal–epithelial transition
Vcam1	Vascular cell adhesion molecule-1
CCR5	Chemokine (C–C motif) receptor 5
VLA-4	Very late antigen-4

Introduction

Carcinogenesis, which is often accompanied by well-orchestrated desmoplastic reactions [1] involving the recruitment of bone marrow-derived mesenchymal stem/stromal cells (MSCs) [2], closely resembles wound healing and scar formation [3]. The stromal component of the tumor microenvironment (TME), which influences the malignant phenotype, consists of three general cell types: cancer-associated fibroblastic cells (CAFs), angiogenic vascular cells, and infiltrating immune cells [4]. However, the precise role of repopulating MSCs in tumorigenesis remains controversial. Khakoo et al. [5] demonstrated that MSCs possess intrinsic, anti-neoplastic properties by inhibiting AKT activity in an E-cadherin-mediated, cell–cell contact-dependent manner. They suggested that MSCs might be useful for treating human malignancies characterized by AKT dysregulation. In contrast, Karnoub et al. [6] demonstrated that the microenvironment of MSCs within the tumor stroma in SCID mice facilitated metastatic spread by paracrine signaling of C–C motif chemokine ligand 5 (CCL5) secreted de novo by MSCs.

Although MSCs are prime candidates for cell-based therapies [7, 8] of a variety of diseases, our understanding of the interactions between colorectal cancer cells and MSCs remain limited [9]. Recent studies have focused disproportionately on the protumorigenic role of MSCs in colorectal cancer growth by differentiation into CAFs, secretion of key paracrine factors, or induction of epithelial–mesenchymal transition (EMT) [10–16]. Tsai et al. [10] and Shinagawa et al. [11] reported that MSCs differentiate into CAFs in the tumor stroma. Similarly, De Boeck et al. [12] showed that MSCs are a source of tumor-associated mesenchymal cells that drive tumor progression. In contrast, Lin et al. [13] isolated MSC-like cells from colon cancer tissues and demonstrated the importance of interleukin-6 (IL-6)/Notch-1/CD44 signals in colon cancer progression. Liu et al. [14] reported that stimulated MSCs in the TME express higher levels of vascular endothelial growth factor (VEGF), which enhances tumor angiogenesis. Furthermore, Li et al. [15] emphasized that such protumorigenic effects of MSCs induced activation of β-catenin signaling. Finally, Mele et al. [16] reported that MSCs induce EMT in human colorectal cancer cells.

Therefore, our goal was to determine whether MSCs could supply contextual signals that promote or suppress colorectal tumor progression. To this end, we hypothesized that MSCs could create a cancer cell niche to modulate colorectal cancer growth in a context-dependent manner. Consequently, we found that MSCs could have multiple effects on colon cancer progression in the xenograft model. Such knowledge of the multifaceted roles of MSCs in colorectal cancer is important before exploring the broader clinical applications of MSC-based therapies.

Methods

For detailed Methods, please refer to the Supplementary Material.

Experimental animals

Lewis rats were purchased from Charles River Laboratories Japan (Yokohama, Japan). SD-TG [CAG-enhanced green fluorescence protein (eGFP)] rats [17] and athymic nude (BALB/cSlc-nu/nu) mice were obtained from Japan SLC

Inc. (Hamamatsu, Japan). NOG mice (NOD/Shi-scid, IL-2Rγnull) [18] were purchased from the Central Institute of Experimental Animals (Kawasaki, Japan). FOX CHASE SCID C.B-17/lcr-scid/scidJcl mice were from CLEA Japan Inc. (Tokyo, Japan). All animal studies were performed under the supervision of the Committee for Animal Research Center of Sapporo Medical University and in accordance with protocols approved by the Institutional Animal Care and Use Committee. The animals were maintained according to the guidelines of the University Committee for Animal Research.

Cell lines and culture conditions

The six human colorectal cancer cell lines used in the xenograft experiments were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA), RIKEN BioResource Center (Tsukuba, Japan), or the Japanese Collection of Research Bioresources (Osaka, Japan). All cell lines, LoVo (DSMZ ACC 350), HCT116 (ATCC: CCL 247), COLO 320 (DSMZ ACC 144), DLD-1 (ATCC: CCL 221), HCT-15 (ATCC: CCL 225), and HT-29 (DSMZ ACC 299), were cultured under conditions recommended by their supplier.

Isolation and culture of rat MSCs

Briefly, bone marrow cells were harvested by inserting a needle into the shafts of femurs and tibiae, and flushing with 30 mL complete α-modified Eagle's medium (αMEM) containing 20 % fetal bovine serum (FBS). Cell suspensions were filtered through a 70-μm nylon filter (Becton–Dickinson, Franklin Lakes, NJ, USA) and seeded in 75 cm^2 flasks. The cells were grown in complete αMEM containing 20 % FBS at 37 °C in a humidified atmosphere with 5 % CO_2. After 3 days, the medium was replaced with fresh medium containing 10 % FBS, and the adherent cells were grown to 80 % confluence for passage 0. In accordance with the criteria of the International Society for Cellular Therapy [19], cells at passage 3–5 were used in subsequent experiments. eGFP-labeled MSCs were harvested from CAG-eGFP rats and cultured as described above [20]. Immunophenotyping and in vitro differentiation capacity of MSCs were determined as described in Supplementary Material.

Xenograft model and evaluation of tumor growth

For xenograft experiments, viable colon cancer cells were inoculated alone or together with rat GFP-labeled MSCs by subcutaneous injection into recipient NOG, SCID, and nude mice. Animals were observed daily, and tumor volumes were measured in two dimensions three times weekly using precision calipers. The tumor volume was calculated and expressed as the mean ± SEM from five mice in each group as described previously [21]. The two most distinctive cell lines underwent subsequent xenograft analyses using nude, SCID, and NOG mice, namely, COLO 320 as an MSC-dependent cell line and HT-29 as an MSC-independent cell line as described above. The ratio of MSCs to colon cancer cells was 1:1, unless otherwise indicated, as described elsewhere in detail (Supplementary Fig. 1a, b).

RNA isolation and quantitative real-time reverse transcription PCR (qRT-PCR) analysis

Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany), and 500 ng of total RNA was reverse transcribed into cDNA with oligo-dT primers using SuperScriptIII Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). The primers are described in Supplementary Table 1. Human *GAPDH* and rat *Gapdh* primers were used as an internal standard for the integrity and quantity of RNA. Quantitative real-time reverse transcription PCR (qRT-PCR) was performed using a GeneAmp 7000 Sequence Detection System (Applied Biosystems) with TaqMan Universal PCR Master Mix (Applied Biosystems) for 40 cycles of a two-step PCR amplification protocol (95 °C for 15 s and 60 °C for 1 min). Data were analyzed using the $^{\Delta\Delta}C_t$ method [22]. qRT-PCR was performed in triplicate unless otherwise indicated.

Evaluation of tumor microvessel density

Tumor microvessel density (MVD) (microvessel/mm^2) of the entire tumor area was evaluated using a previously described method [23]. Two independent observers counted the number of microvessels in the entire field of the tumor. MVD of specimens was calculated as the mean MVD of triplicates.

Characterization of eGFP-labeled rat MSC-derived cells in xenografts

eGFP fluorescence and primary antibodies against thymus cell antigen-1 (Thy-1, CD90), neural/glial antigen 2 (NG2) chondroitin sulfate proteoglycan, CD31, von Willebrand factor, α-smooth muscle actin (αSMA), desmin, vimentin, and RM-4 [24] (Supplementary Table 2) were used for immunofluorescence analyses together with Alexa Fluor 594-labeled secondary antibodies.

Two-color fluorescence in situ hybridization (FISH) analysis

Two-color FISH of human colorectal cancer xenografts subcutaneously implanted into SCID mice with or without