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M2 Monocyte Microparticles Are Increased in Intracerebral Hemorrhage

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Abstract

Background—Intracerebral hemorrhage (ICH) is a severe neurologic condition with no proven treatment. Recent evidence suggests that monocytes, a heterogeneous group of cells with M1 and M2 phenotypes, contribute to secondary damage following ICH. Microparticles are vesicles .1–1 μ m in size that are released from cells. We hypothesized that M1 and M2 monocyte microparticles (mMP) would be differentially expressed in ICH cases and controls.

Methods—In a single-center, prospective, observational study, consecutive ICH cases were enrolled within 12 hours of symptom onset. Age (± 5 years)-, race-, and sex-matched controls were recruited. M1 and M2 mMP numbers were determined in plasma samples using flow cytometry and protein biomarkers using standardized assays. The Mann–Whitney *U* test compared M1 and M2 mMP counts between cases and controls. Standardized regression coefficients compared M1 and M2 mMP with C-reactive protein (CRP) and serum amyloid A (SAA).

Results—Nineteen ICH case-control pairs were enrolled. The median number of M1 mMP was not significantly different between ICH cases (8.63×10^7 /milliliter (mL)) compared with controls (8.64×10^7 /mL), ($P = .525$). The median number of M2 mMP was significantly higher in ICH cases (1.61×10^6 /mL) compared with controls (4.46×10^5 /mL) ($P = .027$). There were no significant associations for M1 or M2 mMP with CRP or SAA.

Conclusion—Higher numbers of M2 mMP in ICH cases compared with controls is hypothesis generating. It may represent differences in the chronic inflammatory status in patients susceptible to ICH, such as cellular activation or apoptosis. Further research is needed, including serial plasma samples, to elucidate the pathophysiology of monocytes and mMP following ICH.

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The study was performed at the University of Cincinnati within the Department of Emergency Medicine and the Department of Internal Medicine, Division of Nephrology.

Keywords

Stroke; intracerebral hemorrhage; neuroinflammation; monocytes; microparticles; microvesicles

Introduction

Intracerebral hemorrhage (ICH), a type of stroke caused by bleeding within the brain, is a severe neurologic disorder¹ that affects up to 67,000 people annually in the United States.² It accounts for 10% of strokes, but 50% of stroke mortality,³ with a 30-day case-fatality of approximately 40%.⁴ Those who survive often have significant morbidity.⁵ There are no medical or surgical therapies that have shown definitive benefit in ICH. The hemorrhage itself causes a primary injury to the brain, which is followed by a secondary inflammatory injury that contributes to poorer outcomes. White blood cells (WBCs) play an important role in this inflammatory damage,^{6,7} and increasing evidence suggests that monocytes, a specific type of WBC, are particularly important.^{8–12} We previously found in 2 independent cohorts of ICH patients that higher monocyte count was associated with 30-day case-fatality.^{10,11} Higher levels of chemokine C-C motif ligand 2 (CCL2), the dominant cytokine for monocyte recruitment, were independently associated with poor functional outcome after ICH.⁹ In a recent investigation of 1302 ICH patients, higher admission monocyte count was an independent predictor of ICH hematoma expansion.¹³ Preclinical studies have also found that monocytes are the main cells recruited to ICH site, and that mice with fewer of these monocytes, or those treated to block the cells, had better outcomes.^{8,9}

Monocytes are a heterogeneous group of cells that include M1 and M2 monocyte/macrophage subtypes with distinct actions.¹⁴ M1 cells participate in acute inflammatory pathways,^{15,16} whereas M2 macrophages are thought to primarily secrete anti-inflammatory mediators in the setting of acute inflammation.^{17,18} A higher M1/M2 ratio thus implies an environment that is more inflammatory and oxidative, whereas a lower M1/M2 ratio suggests an environment for repair and regeneration.¹⁹

Microparticles (MP), also known as microvesicles, are vesicles released from the plasma membrane of cells and are .1–1 μ m in size.²⁰ Research interest in these extracellular particles has dramatically increased over the past decade, in part due to more understanding about their multiple potential functions such as cellular communication, coagulation, and inflammation.²¹ Monocyte microparticles (mMP) are released in vivo as a result of cellular activation or apoptosis and can play a role in inflammation,²⁰ translocation of transcription factors,²² coagulation,^{20,23} and vascular inflammation.^{24,25} mMP have been studied in a variety of disease states,^{22,26–34} but to the best of our knowledge, have not been studied in ICH. In the current investigation, we investigated M1 and M2 mMP in plasma samples from ICH patients and matched controls.

Materials and Methods

Cases were enrolled within 12 hours of ICH symptom onset between August 2013 and May 2014 at an urban, tertiary care hospital. The median time from stroke symptom onset to blood draw for all cases was 6.8 hours (interquartile range 4.5–9.8 hours). Study procedures

were approved by the local Institutional Review Board. Potential cases were identified from pages to the Stroke Team and 24/7 screening of the emergency department, neurology floor beds, and neuroscience intensive care unit by hospital-based research assistants. Inclusion criteria were as follows: age 18 years or older, primary spontaneous ICH with no radiographic features suggestive of aneurysmal subarachnoid hemorrhage, traumatic contusion, vascular malformation, tumor, or venous sinus thrombosis. Exclusion criteria included recent history (<2 weeks) of myocardial infarction, stroke, surgery, or hospitalization, known active malignant cancer, lymphoma, or leukemia, history of a chronic inflammatory disorder, current treatment with anticoagulants, congenital or heritable disorder of coagulation, and liver disease or cirrhosis. The patient or their legally authorized representative provided signed, informed consent, and were then asked about medical history related to stroke risk factors, including history of smoking, diabetes, hypertension, coronary artery disease, atrial fibrillation, and prior stroke. Venipuncture was performed for collection of a venous blood specimen.

Controls were matched based on age (± 5 years), race, and gender, and were recruited from hospital staff and well visitors to the hospital using the same inclusion and exclusion criteria.

Specimen Collection and Processing

Seven milliliters of venous blood was drawn into trace metal (Kendall Monoject, Minneapolis, MN) heparin tubes. Within 2 hours, the mixture was centrifuged at room temperature for 10 minutes at $2000 \times g$. The supernatant plasma was aliquoted into cryovials and frozen at -80°C until the time of laboratory analysis. Levels of the following cytokines were determined using commercially available multiplex assays (Cytokine 30-Plex Human Panel, ThermoFisher Scientific, Waltham, MA): interleukin-4 (IL-4), IL-6, IL-8, IL-10, Monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor alpha (TNF-alpha). Adiponectin and serum amyloid A (SAA) were tested using an individual enzyme-linked immunosorbent assay (Abcam, Cambridge, United Kingdom) for each. C-reactive protein (CRP) level was determined in the clinical laboratory. Adiponectin is an adipokine that has been shown to reduce the production of mMP²⁴ and to push monocytes toward the M2 anti-inflammatory phenotype.³⁵ IL-4 and IL-10 are also anti-inflammatory biomarkers and are secreted by M2 monocytes.^{17,18} TNF-alpha, IL-6, IL-8, and MCP-1 are inflammatory biomarkers reported to participate in pathways of activation with M1 inflammatory monocytes.^{15,16} SAA is a biomarker for the inflammatory acute phase response,³⁶ and CRP is a marker of inflammation that is used very commonly in the clinical setting currently.³⁷

Flow Cytometry

To 50 μL of plasma, 5 μL of CD14 PE and 5 μL of CD16 PECy7 (Biolegend, San Diego, CA) were added. Simultaneously, compensation beads (eBioscience, San Diego, CA) were incubated with the antibodies. Isotype controls for the antibodies of interest (IgG1 k, PE and IgG1 k PECy7) were included. Samples were analyzed by flow cytometry (BD Biosciences, LSR II, San Jose, CA). The instrument was cleaned before analyzing each set of samples. Filtered water, phosphate-buffered saline, and antibodies alone were also tested to confirm elimination of background. Size beads (sub-micron particle size reference kit, ThermoFisher

Scientific, .1, .2, .5 and 1 μm) functioned as size markers, and a log scale for forward scatter and side scatter parameters was used. Flow cytometry gates were set at $<1 \mu\text{m}$ and $.2 \mu\text{m}$. Flow analysis was performed with FlowJo software (FlowJo, LLC, Ashland, OR).

Statistical Analysis

Study data were collected and managed using REDCap (Research Electronic Data Capture, Nashville, TN) electronic data capture tools hosted at Cincinnati Children's Hospital Medical Center. REDCap is a secure, web-based application designed to support data capture for research studies.³⁸ All statistical analyses were conducted using SPSS 24.0 (IBM SPSS Statistics for Windows, IBM Corporation, Armonk, NY) and R-3.2.4; base package. Graphics were created using R-3.2.4; base package and beeswarm.³⁹ The median number of M1 and M2 mMP was compared between ICH cases and controls using the Mann–Whitney *U* test. The mMP data were transformed using the natural log function to achieve better distribution. Regression coefficients were calculated for M1 and M2 mMP with the inflammatory markers CRP and SAA.

Results

Nineteen ICH case-control pairs were enrolled. The mean age for ICH cases was 67 years (standard deviation 14 years), for ICH controls 66 years (standard deviation 13 years), and for both case and control groups, 47% were African American and 68% were women (Table 1). The absolute number of M1 (CD14+CD16–) and M2 (CD14+CD16+) mMP were determined in the plasma samples as well as the levels of protein biomarkers of interest (Table 2).

The median number of M1 (CD14+CD16–) mMP was not significantly different between ICH cases ($8.64 \times 10^7/\text{mL}$) compared with ICH controls ($8.63 \times 10^7/\text{mL}$, $P = .525$; Fig 1). The median number of M2 (CD14+CD16+) mMP was significantly higher in ICH cases ($1.61 \times 10^6/\text{mL}$) compared with controls ($4.46 \times 10^5/\text{mL}$, $P = .027$; Fig 2). Standardized regression coefficients were not statistically significant for M1 mMP and CRP in ICH cases (Beta $-.218$, $P = .369$) and controls (Beta $.297$, $P = .216$), and for M1 mMP and SAA in ICH cases (Beta $.003$, $P = .992$) and controls (Beta $-.248$, $P = .307$). Likewise, regression coefficients were not statistically significant for M2 mMP and CRP in ICH cases (Beta $-.002$, $P = .994$) and controls (Beta $.237$, $P = .328$), and for M2 mMP and SAA in ICH cases (Beta $-.079$, $P = .748$) and controls (Beta $-.144$, $P = .557$). There were no significant differences for the protein biomarkers of interest in ICH cases and controls (Table 2).

Discussion

We found that the number of M2 mMP was significantly higher in plasma samples from ICH cases compared with controls. M2 monocytes have been reported as anti-inflammatory cells in acute inflammation.^{17,18} Conversely, we also found, in those same ICH cases, that the anti-inflammatory M2-associated cytokines, adiponectin, IL-4, and IL-10, were not increased (Table 2). This apparent contradiction is potentially explained by differences in chronic versus acute inflammation, the timing of blood draw in our study, and the uncertainty whether the increase in M2 mMP resulted from M2 monocyte activation or

apoptosis. Our study included a single plasma sample drawn soon after ICH symptom onset, and thus did not allow for comparison of mMP and cytokines over time. Clinical studies by our group and others have contributed to an increasing body of evidence that monocytes play an important role in the secondary inflammatory injury after ICH.^{10,11,13} Preclinical studies have also supported a specific role for monocytes, as they were the major cell recruited to ICH site in a murine model, and mice with attenuation of monocyte number or activity had better functional outcomes after ICH.^{8,9} mMP are released in vivo as a result of cellular activation or apoptosis, and they can have proinflammatory effects primarily through activation of endothelial cells, fibroblasts, smooth muscle cells, and autocrine effects on other monocytes.²⁰

Our finding that M2 mMP were higher in ICH cases could represent chronic inflammatory differences in these patients compared with controls. Specifically, those patients predisposed to ICH have more long-term activation of M2 monocytes, with resultant higher M2 mMP. Alternatively, higher M2 mMP could be due to more apoptosis of these cells. Our finding of higher M2 mMP, but a lack of increase in M2 associated cytokines, seems contradictory if the mMP are a result of cellular activation. If increased M2 mMP levels are instead due to increased M2 monocyte apoptosis,⁴⁰ our results would be plausible because apoptotic cells would not produce their expected cytokines, and apoptosis is a known major mechanism for MP release. Although we are unable to more fully evaluate cellular activation versus apoptosis in our exploratory study, prior research has identified markers of cellular activation and apoptosis for endothelial cell MPs.⁴¹ More research is warranted regarding similar markers in mMP.

Although it cannot be excluded that the higher levels of M2 mMP in our ICH cases are secondary to dynamic changes immediately following the hemorrhage, given the short time from ICH symptom onset to blood draw, with a median time of 6.8 hours, it seems more likely that the difference is representative of chronic inflammatory status. Obtaining serial plasma samples for each ICH patient, as opposed to the single sample in the current study, would be beneficial to elucidate the development of such changes in monocyte function and expression over time.

The anti-inflammatory cytokines IL-4, IL-10, and TGF-Beta, have been shown to dampen inflammation and promote tissue repair.^{17,18} Adiponectin reduced mMP production²⁴ and promoted the M2 anti-inflammatory monocyte phenotype.³⁵ In contrast to acute inflammation, in which M2 are anti-inflammatory, there are many reports that M2 cells are instead increased and potentially pro-inflammatory in chronic conditions.^{42–52} Similarly, we hypothesize that, in our study, chronic changes in M2 monocyte activity resulted in more M2 mMP in ICH cases.

We did not find a significant difference in the number of M1 mMP in ICH cases compared with controls. We also measured M1-associated inflammatory cytokines including IL-6, IL-8, MCP-1, and TNF-alpha, and, similarly, did not find significant differences (Table 2). M1 monocytes are pro-inflammatory in acute inflammation,^{15,16,19} and would be anticipated to be more active in ICH cases as the inflammatory reaction develops following the hemorrhage. However, the nature and time course of such activation is difficult to predict

based on current knowledge. Future studies should trend changes in mMP levels and inflammatory cytokines over a longer time than the current investigation. The importance of our measured inflammatory biomarkers has been noted in prior monocyte-related studies. M1 monocytes participated in feedback loops with other leukocytes through TNF-alpha, IL-6, IL-12, and IL-23,^{15,16} and overall created a damaging inflammatory environment. mMP also increased production of proinflammatory cytokines like IL-6, IL-8, and MCP-1, and induced brain endothelial cells to produce endothelial MPs.⁵³

The number of M2 mMP did not significantly correlate in either ICH cases or controls with SAA or CRP, 2 common measures of inflammation.^{36,54} As noted for other protein biomarkers in this study, it is plausible that SAA and CRP levels would change more over time following ICH. Based on our results, though, M2 mMP are a potential unique marker of inflammation in the early period following ICH, and representative of a difference not reflected by the more traditional inflammatory biomarkers SAA and CRP.

Strengths of the presented research include a novel report of mMP in both ICH cases and controls. This exploratory work contributes to the rapidly growing MP literature, as well as to the role of monocytes in the secondary inflammatory injury after ICH. To our knowledge, this is the first report of mMP in ICH.

Limitations of the study include the small sample size, the testing of plasma samples from only a single draw instead of serial samples, and the use of only a single method (i.e., conventional flow cytometry) for the determination of mMP levels. Knowledge regarding MP is limited, including their size, morphology, and biochemical composition.⁵⁵ There has been substantial controversy regarding the optimal technique for MP analysis.^{56,57} Although conventional flow cytometry is widely available, easy to access, and is historically the most common technique for MP research, there are a number of potential limitations, including the resolution of the device compared to the size of MP,⁵⁸ reproducibility of results, and the ability to distinguish between 2 or more MP of similar sizes.⁵⁹ In our study, cognizant of these limitations, we performed additional testing with commercial beads and liposomes of various sizes for increased standardization of flow cytometry MP methodology. Other techniques are available for MP analysis but also include notable limitations.⁵⁵ Recent findings have suggested that imaging flow cytometry is a promising method for MP research and worthy of further research.^{21,60}

Conclusion

The current study complements a growing body of literature regarding M1 and M2 monocyte subtypes and mMP in various disease states. Our novel investigation, the first to study mMP in ICH, and the first to report mMP with M1 and M2 classifications, is hypothesis generating. We believe the higher number of M2 mMP in ICH cases compared with controls likely reflects differences in the chronic inflammatory state of monocytes in patients prone to ICH. The lack of correlation of M2 mMP with anti-inflammatory cytokines suggests that M2 monocyte apoptosis or reduced cellular activation may be occurring. Further studies in ICH are needed, including larger sample sizes, serial blood samples, study of whole cell monocytes simultaneously with mMP, and validation methodology such as

imaging flow cytometry in addition to conventional flow cytometry. Additional work in this area has the potential to provide valuable knowledge regarding the pathophysiology, mechanistic role of monocytes, and treatment targets of ICH.

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Figure 1. Box plot and dot plot showing the distribution of CD14+CD16- M1 monocyte microparticles (mMP) for ICH cases and controls. Data were transformed using the natural log function.

Figure 2. Box plot and dot plot showing the distribution of CD14+CD16+ M2 monocyte microparticles (mMP) for ICH cases and controls. Data were transformed using the natural log function.

Table 1

Demographic information and past medical history for intracerebral hemorrhage (ICH) cases and controls

	ICH cases (n = 19)	ICH controls(n = 19)
Age—mean (SD)	67 (14)	66 (13)
Race—n (%)		
African American	9 (47.4)	9 (47.4)
White	9 (47.4)	9 (47.4)
Asian	1 (5.3)	1 (5.3)
Sex—n (%)		
Female	13 (68.4)	13 (68.4)
Male	6 (31.6)	6 (31.6)
Smoking status—n (%)		
Former	8 (42.1)	8 (42.1)
Current	3 (15.8)	0 (.0)
Never	8 (42.1)	11 (57.9)
Prior medical history—n (%)		
Hypertension	13 (68.4)	11 (57.9)
Coronary artery disease	0 (.0)	1 (5.3)
Congestive heart failure	1 (5.3)	0 (.0)
Carotid artery occlusion	0 (.0)	0 (.0)
Intracranial artery stenosis	0 (.0)	0 (.0)
Atrial fibrillation	1 (5.3)	2 (10.5)
Other heart arrhythmia	2 (10.5)	0 (.0)
COPD, asthma	4 (21.1)	2 (10.5)
Diabetes mellitus	7 (36.8)	1 (5.3)
Previous ischemic stroke	2 (10.5)	0 (.0)
Previous hemorrhagic stroke	1 (5.3)	0 (.0)
Resolved/cured malignancy	4 (21.1)	5 (26.3)
Sickle cell disease/Thalassemia/other blood disease	0 (.0)	0 (.0)

Abbreviations: COPD, chronic obstructive pulmonary disease; SD, standard deviation.

Table 2

Median levels of monocyte microparticles and biomarkers of interest for ICH cases and controls

	ICH cases (median)	ICH controls (median)	Difference	<i>P</i> value
M1 microparticles	8.63×10^7	8.64×10^7	4.98×10^4	.511
M2 microparticles	1.61×10^6	4.46×10^5	1.16×10^5	.028
Adiponectin (ug/mL)	47.96	76.47	28.51	.099
CRP (mg/dL)	.40	.70	.30	.189
IL-4 (pg/mL)	26.13	28.42	2.29	.629
IL-6 (pg/mL)	17.41	12.04	5.38	.328
IL-8 (pg/mL)	36.08	28.63	7.45	.274
IL-10 (pg/mL)	13.74	46.04	32.30	.034
MCP-1 (pg/mL)	503.23	491.25	11.98	.782
SAA (ng/mL)	25.82	29.38	3.57	.511
TNF-alpha (pg/mL)	17.55	63.82	46.27	.033

Abbreviations: CRP, C-reactive protein; ICH, intracerebral hemorrhage; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; SAA, serum amyloid A; TNF-alpha, tumor necrosis factor alpha.

From top: CD14+CD16- M1 monocyte microparticles (reported as absolute number per milliliter), CD14+CD16+ M2 monocyte microparticles (absolute number per milliliter).

Protein biomarkers of interest: adiponectin, C-reactive protein, IL-4, IL-6, IL-8, IL-10, monocyte chemoattractant protein-1, serum amyloid A, tumor necrosis factor-alpha.

Adjustment for multiple comparisons for the 9 protein biomarkers: .05/9 = significant *P* value < .0056.